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(71) Applicant (for all designated States except US): BIO-CARDIA, INC. [US/US]; 384 Oyster Point Boulevard, #4, South San Francisco, CA 94080 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): WOHLGEMUTH, Jay [US/US]; 664 Hamilton Avenue, Palo Alto, CA 94301 (US). FRY, Kirk [US/US]; 2604 Ross Road, Palo Alto, CA 94303 (US). MATCUK, George [US/US]; 141C Escondido Village, Stanford, CA 94305 (US). ALTMAN, Peter [US/US]; 717 Evelyn Avenue, Albany, CA 94706 (US). PRENTICE, James [US/US]; 120 Dolores Street, San Francisco, CA 94103 (US). PHILLIPS, Julie [US/US]; 1090 Mirador Terrace, Pacifica, CA 94044 (US). LY, Ngoc [US/US]; 2000 Crystal Springs Road 15-14, San Bruno, CA 94066 (US). WOODWARD, Robert [US/US]; 1828 Rheem Court, Pleasanton, CA 94588 (US).

QUERTERMOUS, Thomas [US/US]; 44 El Rey Road, Portola Valley, CA 94028 (US). JOHNSON, Frances [US/US]; 44 El Rey Road, Portola Valley, CA 94028 (US).

(74) Agents: WARD, Michael, R. et al.; Morrison & Foerster LLP, 425 Market Street, San Francisco, CA 94105-2482 (US).

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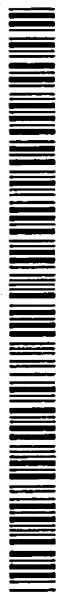
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(54) Title: LEUKOCYTE EXPRESSION PROFILING

(57) Abstract: Leukocyte gene expression profiling is utilized to identify oligonucleotides from gene expression candidate libraries. The expression libraries are generally immobilized on an array. Diagnostic oligonucleotide sets for analysis of leukocyte-related diseases are described.



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LEUKOCYTE EXPRESSION PROFILING

Field of the Invention

This invention is in the field of expression profiling. In particular, this invention is in the field of leukocyte expression profiling.

Background of the Invention

Many of the current shortcomings in diagnosis, prognosis, risk stratification and treatment of disease can be approached through the identification of the molecular mechanisms underlying a disease and through the discovery of nucleotide sequences (or sets of nucleotide sequences) whose expression patterns predict the occurrence or progression of disease states, or predict a patient's response to a particular therapeutic intervention. In particular, identification of nucleotide sequences and sets of nucleotide sequences with such predictive value from cells and tissues that are readily accessible would be extremely valuable. For example, peripheral blood is attainable from all patients and can easily be obtained at multiple time points at low cost. This is a desirable contrast to most other cell and tissue types, which are less readily accessible, or accessible only through invasive and aversive procedures. In addition, the various cell types present in circulating blood are ideal for expression profiling experiments as the many cell types in the blood specimen can be easily separated if desired prior to analysis of gene expression. While blood provides a very attractive substrate for the study of diseases using expression profiling techniques, and for the development of diagnostic technologies and the identification of therapeutic targets, the value of expression profiling in blood samples rests on the degree to which changes in gene expression in these cell types are associated with a predisposition to, and pathogenesis and progression of a disease.

There is an extensive literature supporting the role of leukocytes, e.g., T- and B-lymphocytes, monocytes and granulocytes, including neutrophils, in a wide range of disease processes, including such broad classes as cardiovascular diseases, inflammatory, autoimmune and rheumatic diseases, infectious diseases, transplant rejection, cancer and malignancy, and endocrine diseases. For example, among cardiovascular diseases, such commonly occurring diseases as atherosclerosis, restenosis, transplant vasculopathy and acute coronary syndromes all demonstrate significant T cell involvement (Smith-Norowitz et al. (1999) Clin Immunol 93:168-175; Jude et al. (1994) Circulation 90:1662-8; Belch et al. (1997) Circulation

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95:2027-31). These diseases are now recognized as manifestations of chronic inflammatory disorders resulting from an ongoing response to an injury process in the arterial tree (Ross et al. (1999) Ann Thorac Surg 67:1428-33). Differential expression of lymphocyte, monocyte and neutrophil genes and their products has been demonstrated clearly in the literature. Particularly interesting are examples of differential expression in circulating cells of the immune system that demonstrate specificity for a particular disease, such as arteriosclerosis, as opposed to a generalized association with other inflammatory diseases, or for example, with unstable angina rather than quiescent coronary disease.

A number of individual genes, e.g., CD11b/CD18 (Kassirer et al. (1999) Am Heart J 138:555-9); leukocyte elastase (Amaro et al. (1995) Eur Heart J 16:615-22; and CD40L (Aukrust et al. (1999) Circulation 100:614-20) demonstrate some degree of sensitivity and specificity as markers of various vascular diseases. In addition, the identification of differentially expressed target and fingerprint genes isolated from purified populations of monocytes manipulated in various in vitro paradigms has been proposed for the diagnosis and monitoring of a range of cardiovascular diseases, see, e.g., US Patents Numbers 6,048,709; 6,087,477; 6,099,823; and 6,124,433 "COMPOSITIONS AND METHODS FOR THE TREATMENT AND DIAGNOSIS OF CARDIOVASCULAR DISEASE" to Falb (*see also*, WO 97/30065). Lockhart, in US Patent Number 6,033,860 "EXPRESSION PROFILES IN ADULT AND FETAL ORGANS" proposes the use of expression profiles for a subset of identified genes in the identification of tissue samples, and the monitoring of drug effects.

The accuracy of technologies based on expression profiling for the diagnosis, prognosis, and monitoring of disease would be dramatically increased if numerous differentially expressed nucleotide sequences, each with a measure of specificity for a disease in question, could be identified and assayed in a concerted manner. In order to achieve this improved accuracy, the appropriate sets of nucleotide sequences need to be identified and validated against numerous samples in combination with relevant clinical data. The present invention addresses these and other needs, and applies to any disease or disease state for which differential regulation of genes, or other nucleotide sequences, of peripheral blood can be demonstrated.

Summary of the Invention

The present invention is thus directed to a system for detecting differential gene expression. In one format, the system has one or more isolated DNA molecules

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wherein each isolated DNA molecule detects expression of a gene selected from the group of genes corresponding to the oligonucleotides depicted in the Sequence Listing. It is understood that the DNA sequences and oligonucleotides of the invention may have slightly different sequences than those identified herein. Such sequence variations are understood to those of ordinary skill in the art to be variations in the sequence which do not significantly affect the ability of the sequences to detect gene expression.

The sequences encompassed by the invention have at least 40-50, 50-60, 70-80, 80-85, 85-90, 90-95 % or 95-100% sequence identity to the sequences disclosed herein. In some embodiments, DNA molecules are less than about any of the following lengths (in bases or base pairs): 10,000; 5,000; 2500; 2000; 1500; 1250; 1000; 750; 500; 300; 250; 200; 175; 150; 125; 100; 75; 50; 25; 10. In some embodiments, DNA molecule is greater than about any of the following lengths (in bases or base pairs): 10; 15; 20; 25; 30; 40; 50; 60; 75; 100; 125; 150; 175; 200; 250; 300; 350; 400; 500; 750; 1000; 2000; 5000; 7500; 10000; 20000; 50000. Alternately, a DNA molecule can be any of a range of sizes having an upper limit of 10,000; 5,000; 2500; 2000; 1500; 1250; 1000; 750; 500; 300; 250; 200; 175; 150; 125; 100; 75; 50; 25; or 10 and an independently selected lower limit of 10; 15; 20; 25; 30; 40; 50; 60; 75; 100; 125; 150; 175; 200; 250; 300; 350; 400; 500; 750; 1000; 2000; 5000; 7500 wherein the lower limit is less than the upper limit.

The gene expression system may be a candidate library, a diagnostic agent, a diagnostic oligonucleotide set or a diagnostic probe set. The DNA molecules may be genomic DNA, protein nucleic acid (PNA), cDNA or synthetic oligonucleotides.

In one format, the gene expression system is immobilized on an array. The array may be a chip array, a plate array, a bead array, a pin array, a membrane array, a solid surface array, a liquid array, an oligonucleotide array, a polynucleotide array, a cDNA array, a microfilter plate, a membrane or a chip.

In one format, the genes detected by the gene expression system are selected from the group of genes corresponding to the oligonucleotides depicted in SEQ ID NO:2476, SEQ ID NO: 2407, SEQ ID NO:2192, SEQ ID NO: 2283, SEQ ID NO:6025, SEQ ID NO: 4481, SEQ ID NO:3761, SEQ ID NO: 3791, SEQ ID NO:4476, SEQ ID NO: 4398, SEQ ID NO:7401, SEQ ID NO: 1796; SEQ ID NO:4423, SEQ ID NO: 4429, SEQ ID NO:4430, SEQ ID NO: 4767, SEQ ID NO:4829 and SEQ ID NO: 8091.

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The present invention is further directed to a diagnostic oligonucleotide set for a disease having at least two oligonucleotides wherein the oligonucleotides have a sequence selected from those oligonucleotide sequences listed in Table 2, Table 3, or

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the Sequence Listing which are differentially expressed in leukocytes genes in an individual with at least one disease criterion for at least one leukocyte-related disease as compared to the expression in leukocytes in an individual without the at least one disease criterion, wherein expression of the two or more genes of the gene expression library is correlated with at least one disease criterion.

The present invention is further directed to a diagnostic oligonucleotide set for a disease having at least one oligonucleotide wherein the oligonucleotide has a sequence selected from those sequences listed in Table 2, Table 3, or the sequence listing which is differentially expressed in leukocytes in an individual with at least one disease criterion for a disease selected from Table 1 as compared to leukocytes in an individual without at least one disease criterion, wherein expression of the at least one gene from the gene expression library is correlated with at least one disease criterion, wherein the differential expression of the at least one gene has not previously been described. In one format, two or more oligonucleotides are utilized.

In the diagnostic oligonucleotide sets of the invention the disease criterion may include data selected from patient historic, diagnostic, prognostic, risk prediction, therapeutic progress, and therapeutic outcome data. This includes lab results, radiology results, pathology results such as histology, cytology and the like, physical examination findings, and medication lists.

In the diagnostic oligonucleotide sets of the invention the leukocytes comprise peripheral blood leukocytes or leukocytes derived from a non-blood fluid. The non-blood fluid may be selected from colon, sinus, spinal fluid, saliva, lymph fluid, esophagus, small bowel, pancreatic duct, biliary tree, ureter, vagina, cervix uterus and pulmonary lavage fluid.

In the diagnostic oligonucleotide sets of the invention the leukocytes may include leukocytes derived from urine or a joint biopsy sample or biopsy of any other tissue or may be T-lymphocytes.

In the diagnostic oligonucleotide sets of the invention the disease may be selected from cardiac allograft rejection, kidney allograft rejection, liver allograft rejection, atherosclerosis, congestive heart failure, systemic lupus erythematosus (SLE), rheumatoid arthritis, osteoarthritis, and cytomegalovirus infection.

The diagnostic oligonucleotide sets of the invention may further include one or more cytomegalovirus (CMV) nucleotide sequences, wherein expression of the CMV nucleotide sequence is correlated with CMV infection.

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The diagnostic nucleotide sets of the invention may further include one or more Epstein-Barr virus (EBV) nucleotide sequences, wherein expression of the one or more EBV nucleotide sequences is correlated with EBV infection.

In the present invention, expression may be differential expression, wherein the differential expression is one or more of a relative increase in expression, a relative decrease in expression, presence of expression or absence of expression, presence of disease or absence of disease. The differential expression may be RNA expression or protein expression. The differential expression may be between two or more samples from the same patient taken on separate occasions or between two or more separate patients or between two or more genes relative to each other.

The present invention is further directed to a diagnostic probe set for a disease where the probes correspond to at least one oligonucleotide wherein the oligonucleotides have a sequence ssuch as those listed in Table 2, Table 3, or the Sequence Listing which is differentially expressed in leukocytes in an individual with at least one disease criterion for a disease selected from Table 1 as comapared to leukocytes in an individual without the at least one disease criterion, wherein expression of the oligonucleotide is correlated with at least one disease criterion, and further wherein the differential expression of the at least one nucleotide sequence has not previously been described.

The present invention is further directed to a diagnostic probe set wherein the probes include one or more of probes useful for proteomics and probes for nucleic acids cDNA, or synthetic oligonucleotides.

The present invention is further directed to an isolated nucleic acid having a sequences such as those listed in Table 3B or Table 3C or the Sequence Listing.

The present invention is further directed to polypeptides wherein the polypeptides are encoded by the nucleic acid sequences in Tables 3B, 3C and the Sequence Listing.

The present invention is further directed to a polynucleotide expression vector containing the polynucleotide of Tables 3B-3C or the Sequence Listing in operative association with a regulatory element which controls expression of the polynucleotide in a host cell. The present invention is further directed to host cells transformed with the expression vectors of the invention. The host cell may be prokaryotic or eukaryotic.

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The present invention is further directed to fusion proteins produced by the host cells of the invention. The present invention is further directed to antibodies directed to the fusion proteins of the invention. The antibodies may be monoclonal or polyclonal antibodies.

The present invention is further directed to kits comprising the diagnostic oligonucleotide sets of the invention. The kits may include instructions for use of the kit.

The present invention is further directed to a method of diagnosing a disease by obtaining a leukocyte sample from an individual, hybridizing nucleic acid derived from the leukocyte sample with a diagnostic oligonucleotide set, and comparing the expression of the diagnostic oligonucleotide set with a molecular signature indicative of the presence or absence of the disease.

The present invention is further directed to a method of detecting gene expression by a) isolating RNA and b) hybridizing the RNA to isolated DNA molecules wherein the isolated DNA molecules detect expression of a gene wherein the gene corresponds to one of the oligonucleotides depicted in the Sequence Listing.

The present invention is further directed to a method of detecting gene expression by a) isolating RNA; b) converting the RNA to nucleic acid derived from the RNA and c) hybridizing the nucleic acid derived from the RNA to isolated DNA molecules wherein the isolated DNA molecules detect expression of a gene wherein the gene corresponds to one of the oligonucleotides depicted in the Sequence Listing. In one format, the nucleic acid derived from the RNA is cDNA.

The present invention is further directed to a method of detecting gene expression by a) isolating RNA; b) converting the RNA to cRNA or aRNA and c) hybridizing the cRNA or aRNA to isolated DNA molecules wherein the isolated DNA molecules detect expression of a gene corresponding to one of the oligonucleotides depicted in the Sequence Listing.

The present invention is further directed to a method of monitoring progression of a disease by obtaining a leukocyte sample from an individual, hybridizing the nucleic acid derived from leukocyte sample with a diagnostic oligonucleotide set, and comparing the expression of the diagnostic oligonucleotide set with a molecular signature indicative of the presence or absence of disease progression.

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The present invention is further directed to a method of monitoring the rate of progression of a disease by obtaining a leukocyte sample from an individual, hybridizing the nucleic acid derived from leukocyte sample with a diagnostic oligonucleotide set, and comparing the expression of the diagnostic oligonucleotide set with a molecular signature indicative of the presence or absence of disease progression.

The present invention is further directed to a method of predicting therapeutic outcome by obtaining a leukocyte sample from an individual, hybridizing the nucleic acid derived from leukocyte sample with a diagnostic oligonucleotide set, and comparing the expression of the diagnostic oligonucleotide set with a molecular signature indicative of the predicted therapeutic outcome.

The present invention is further directed to a method of determining prognosis by obtaining a leukocyte sample from an individual, hybridizing the nucleic acid derived from leukocyte sample with a diagnostic oligonucleotide set, and comparing the expression of the diagnostic oligonucleotide set with a molecular signature indicative of the prognosis.

The present invention is further directed to a method of predicting disease complications by obtaining a leukocyte sample from an individual, hybridizing nucleic acid derived from the leukocyte sample with a diagnostic oligonucleotide set, and comparing the expression of the diagnostic oligonucleotide set with a molecular signature indicative of the presence or absence of disease complications.

The present invention is further directed to a method of monitoring response to treatment, by obtaining a leukocyte sample from an individual, hybridizing the nucleic acid derived from leukocyte sample with a diagnostic oligonucleotide set, and comparing the expression of the diagnostic oligonucleotide set with a molecular signature indicative of the presence or absence of response to treatment.

In the methods of the invention the invention may further include characterizing the genotype of the individual, and comparing the genotype of the individual with a diagnostic genotype, wherein the diagnostic genotype is correlated with at least one disease criterion. The genotype may be analyzed by one or more methods selected from the group consisting of Southern analysis, RFLP analysis, PCR, single stranded conformation polymorphism and SNP analysis.

The present invention is further directed to a method of non-invasive imaging by providing an imaging probe for a nucleotide sequence that is differentially

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The present invention is further directed to a control RNA for use in expression profile analysis, where the RNA extracted from the buffy coat samples is from at least four individuals.

The present invention is further directed to a method of RNA preparation suitable for diagnostic expression profiling by obtaining a leukocyte sample from a subject, adding actinomycin-D to a final concentration of 1 ug/ml, adding cycloheximide to a final concentration of 10 ug/ml, and extracting RNA from the leukocyte sample. In the method of RNA preparation of the invention the actinomycin-D and cycloheximide may be present in a sample tube to which the leukocyte sample is added. The method may further include centrifuging the sample at 4°C to separate mononuclear cells.

The present invention is further directed to a method of identifying at least one diagnostic probe set for assessing atherosclerosis by (a) providing a library of candidate oligonucleotides, which candidate oligonucleotides are differentially expressed in leukocytes which are undergoing adhesion to an endothelium relative to their expression in leukocytes that are not undergoing adhesion to an endothelium; (b) assessing expression of two or more oligonucleotides, which two or more oligonucleotides correspond to components of the library of candidate oligonucleotides, in a subject sample of leukocytes; (c) correlating expression of the two or more oligonucleotides with at least one criterion, which criterion includes one

or more indicators of adhesion to an endothelium; and, (d) recording the molecular signature in a database.

The present invention is further directed to a method of identifying at least one diagnostic probe set for assessing atherosclerosis by (a) providing a library of candidate oligonucleotides, which candidate oligonucleotides are differentially expressed in leukocytes which are undergoing adhesion to an endothelium relative to their expression in leukocytes that are not undergoing adhesion to an endothelium; (b) assessing expression of two or more oligonucleotides, which two or more oligonucleotides correspond to components of the library of candidate nucleotide sequences, in a subject sample of epithelial cells; (c) correlating expression of the two or more nucleotide sequences with at least one criterion, which criterion comprises one or more indicator of adhesion to an endothelium; and (d) recording the molecular signature in a database.

The present invention is further directed to methods of leukocyte expression profiling including methods of analyzing longitudinal clinical and expression data. The rate of change and/or magnitude and direction of change of gene expression can be correlated with disease states and the rate of change of clinical conditions/data and/or the magnitude and direction of changes in clinical data. Correlations may be discovered by examining these expression or clinical changes that are not found in the absence of such changes.

The present invention is further directed to methods of leukocyte profiling for analysis and/or detection of one or more viruses. The virus may be CMV, HIV, hepatitis or other viruses. Both viral and human leukocyte genes can be subjected to expression profiling for these purposes.

Brief Description of the Sequence Listing

The table below gives a description of the sequence listing. There are 8830 entries. The Sequence Listing presents 50mer oligonucleotide sequences derived from human leukocyte, plant and viral genes. These are listed as SEQ IDs 1-8143. The 50mer sequences and their sources are also displayed in Table 8. Most of these 50mers were designed from sequences of genes in Tables 2, 3A, B and C and the Sequence listing.

SEQ IDs 8144-8766 are the cDNA sequences derived from human leukocytes that were not homologous to UniGene sequences or sequences found in dbEST at the

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time they were searched. Some of these sequences match human genomic sequences and are listed in Tables 3B and C. The remaining clones are putative cDNA sequences that contained less than 50% masked nucleotides when submitted to RepeatMasker, were longer than 147 nucleotides, and did not have significant similarity to the UniGene Unique database, dbEST, the NR nucleotide database of Genbank or the assembled human genome of Genbank.

SEQ IDs 8767-8770, 8828-8830 and 8832 are sequences that appear in the text and examples (primer, masked sequences, exemplary sequences, etc.).

SEQ IDs 8771-8827 are CMV PCR primers described in Example 17.

Brief Description of the Figures

Figure 1: Figure 1 is a schematic flow chart illustrating a schematic instruction set for characterization of the nucleotide sequence and/or the predicted protein sequence of novel nucleotide sequences.

Figure 2: Figure 2 depicts the components of an automated RNA preparation machine.

Figure 3: Figure 3 describes kits useful for the practice of the invention. Figure 3A describes the contents of a kit useful for the discovery of diagnostic nucleotide sets. Figure 3B describes the contents of a kit useful for the application of diagnostic nucleotide sets.

Figure 4 shows the results of six hybridizations on a mini array graphed ($n=6$ for each column). The error bars are the SEM. This experiment shows that the average signal from AP prepared RNA is 47% of the average signal from GS prepared RNA for both Cy3 and Cy5.

Figure 5 shows the average background subtracted signal for each of nine leukocyte-specific genes on a mini array. This average is for 3-6 of the above-described hybridizations for each gene. The error bars are the SEM.

Figure 6 shows the ratio of Cy3 to Cy5 signal for a number of genes. After normalization, this ratio corrects for variability among hybridizations and allows comparison between experiments done at different times. The ratio is calculated as the Cy3 background subtracted signal divided by the Cy5 background subtracted signal. Each bar is the average for 3-6 hybridizations. The error bars are SEM.

Figure 7 shows data median Cy3 background subtracted signals for control RNAs using mini arrays.

Figure 8 shows data from an array hybridization.

Figure 9 shows a comparison of gene expression in samples obtained from cardiac transplant patients with low rejection grade and high rejection grade.

Figure 10 shows differential gene expression between samples from patients with grade 0 and grade 3A rejection.

Brief Description of the Tables

Table 1: Table 1 lists diseases or conditions amenable to study by leukocyte profiling.

Table 2: Table 2 describes genes and other nucleotide sequences identified using data mining of publically available publication databases and nucleotide sequence databases. Corresponding Unigene (build 133) cluster numbers are listed with each gene or other nucleotide sequence.

Table 3A: Table 3A describes 48 clones whose sequences align to two or more non-contiguous sequences on the same assembled human contig of genomic sequence. The Accession numbers are from the March 15, 2001 build of the human genome. The file date for the downloaded data was 4/17/01. The alignments of the clone and the contig are indicated in the table. The start and stop offset of each matching region is indicated in the table. The sequence of the clones themselves is included in the sequence listing. The alignments of these clones strongly suggest that they are novel nucleotide sequences. Furthermore, no EST or mRNA aligning to the clone was found in the database. These sequences may prove useful for the prediction of clinical outcomes.

Table 3B: Table 3B describes Identified Genomic Regions that code for novel mRNAs. The table contains 591 identified genomic regions that are highly similar to the cDNA clones. Those regions that are within ~100 to 200 Kb of each other on the same contig are likely to represent exons of the same gene. The indicated clone is exemplary of the cDNA clones that match the indicated genomic region. The "number clones" column indicates how many clones were isolated from the libraries that are similar to the indicated region of the chromosome. The probability number is the likelihood that region of similarity would occur by chance on a random sequence. The Accession numbers are from the March 15, 2001 build of the human genome. The file date for the downloaded data was 4/17/01. These sequences may prove useful for the prediction of clinical outcomes.

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Table 3C: Table 3C describes differentially expressed nucleotide sequences useful for the prediction of clinical outcomes. This table contains 4517 identified cDNAs and cDNA regions of genes that are members of a leukocyte candidate library, for use in measuring the expression of nucleotide sequences that could subsequently be correlated with human clinical conditions. The regions of similarity were found by searching three different databases for pair wise similarity using blastn. The three databases were UniGene Unique build 3/30/01, file Hs.seq.uniq.Z; the downloadable database at ftp.ncbi.nlm.nih.com/blast/db/est human.Z with date 4/8/01 which is a section of Genbank version 122; and the non-redundant section of Genbank ver 123. The Hs.XXXXXX numbers represent UniGene accession numbers from the Hs.seq.uniq.Z file of 3/30/01. The clone sequences are not in the sequence listing.

Table 4: Table 4 describes patient groups and diagnostic gene sets

Table 5: Table 5 describes the nucleotide sequence databases used in the sequence analysis described herein.

Table 6: Table 6 describes the algorithms and software packages used for exon and polypeptide prediction used in the sequence analysis described herein.

Table 7: Table 7 describes the databases and algorithms used for the protein sequence analysis described herein.

Table 8: Table 8 describes leukocyte probes spotted on the microarrays.

Table 9: Table 9 describes Cardiac Transplant patient RNA samples and array hybridizations.

Table 10: Table 10 describes differentially expressed probes identified when comparing leukocyte expression profiles obtained from high and low grade cardiac transplant rejection patients.

Detailed Description of the Invention

Definitions

Unless defined otherwise, all scientific and technical terms are understood to have the same meaning as commonly used in the art to which they pertain. For the purpose of the present invention, the following terms are defined below.

In the context of the invention, the term "gene expression system" refers to any system, device or means to detect gene expression and includes diagnostic agents, candidate libraries, oligonucleotide sets or probe sets.

The term "diagnostic oligonucleotide set" generally refers to a set of two or more oligonucleotides that, when evaluated for differential expression of their products, collectively yields predictive data. Such predictive data typically relates to diagnosis, prognosis, monitoring of therapeutic outcomes, and the like. In general, the components of a diagnostic oligonucleotide set are distinguished from nucleotide sequences that are evaluated by analysis of the DNA to directly determine the genotype of an individual as it correlates with a specified trait or phenotype, such as a disease, in that it is the pattern of expression of the components of the diagnostic nucleotide set, rather than mutation or polymorphism of the DNA sequence that provides predictive value. It will be understood that a particular component (or member) of a diagnostic nucleotide set can, in some cases, also present one or more mutations, or polymorphisms that are amenable to direct genotyping by any of a variety of well known analysis methods, e.g., Southern blotting, RFLP, AFLP, SSCP, SNP, and the like.

A "disease specific target oligonucleotide sequence" is a gene or other oligonucleotide that encodes a polypeptide, most typically a protein, or a subunit of a multi-subunit protein, that is a therapeutic target for a disease, or group of diseases.

A "candidate library" or a "candidate oligonucleotide library" refers to a collection of oligonucleotide sequences (or gene sequences) that by one or more criteria have an increased probability of being associated with a particular disease or group of diseases. The criteria can be, for example, a differential expression pattern in a disease state or in activated or resting leukocytes in vitro as reported in the scientific or technical literature, tissue specific expression as reported in a sequence database, differential expression in a tissue or cell type of interest, or the like. Typically, a candidate library has at least 2 members or components; more typically, the library has in excess of about 10, or about 100, or about 1000, or even more, members or components.

The term "disease criterion" is used herein to designate an indicator of a disease, such as a diagnostic factor, a prognostic factor, a factor indicated by a medical or family history, a genetic factor, or a symptom, as well as an overt or confirmed diagnosis of a disease associated with several indicators such as those selected from the above list. A disease criterion includes data describing a patient's health status, including retrospective or prospective health data, e.g. in the form of the

patient's medical history, laboratory test results, diagnostic test result, clinical events, medications, lists, response(s) to treatment and risk factors, etc.

The terms "molecular signature" or "expression profile" refers to the collection of expression values for a plurality (e.g., at least 2, but frequently about 10, about 100, about 1000, or more) of members of a candidate library. In many cases, the molecular signature represents the expression pattern for all of the nucleotide sequences in a library or array of candidate or diagnostic nucleotide sequences or genes. Alternatively, the molecular signature represents the expression pattern for one or more subsets of the candidate library. The term "oligonucleotide" refers to two or more nucleotides. Nucleotides may be DNA or RNA, naturally occurring or synthetic.

The term "healthy individual," as used herein, is relative to a specified disease or disease criterion. That is, the individual does not exhibit the specified disease criterion or is not diagnosed with the specified disease. It will be understood, that the individual in question, can, of course, exhibit symptoms, or possess various indicator factors for another disease.

Similarly, an "individual diagnosed with a disease" refers to an individual diagnosed with a specified disease (or disease criterion). Such an individual may, or may not, also exhibit a disease criterion associated with, or be diagnosed with another (related or unrelated) disease.

An "array" is a spatially or logically organized collection, e.g., of oligonucleotide sequences or nucleotide sequence products such as RNA or proteins encoded by an oligonucleotide sequence. In some embodiments, an array includes antibodies or other binding reagents specific for products of a candidate library.

When referring to a pattern of expression, a "qualitative" difference in gene expression refers to a difference that is not assigned a relative value. That is, such a difference is designated by an "all or nothing" valuation. Such an all or nothing variation can be, for example, expression above or below a threshold of detection (an on/off pattern of expression). Alternatively, a qualitative difference can refer to expression of different types of expression products, e.g., different alleles (e.g., a mutant or polymorphic allele), variants (including sequence variants as well as post-translationally modified variants), etc.

In contrast, a "quantitative" difference, when referring to a pattern of gene expression, refers to a difference in expression that can be assigned a value on a

graduated scale, (e.g., a 0-5 or 1-10 scale, a + - +++ scale, a grade 1- grade 5 scale, or the like; it will be understood that the numbers selected for illustration are entirely arbitrary and in no-way are meant to be interpreted to limit the invention).

Gene Expression Systems of the Invention

The invention is directed to a gene expression system having one or more oligonucleotides wherein the one or more oligonucleotides has a nucleotide sequence which detects expression of a gene corresponding to the oligonucleotides depicted in the Sequence Listing. In one format, the oligonucleotide detects expression of a gene that is differentially expressed in leukocytes. The gene expression system may be a candidate library, a diagnostic agent, a diagnostic oligonucleotide set or a diagnostic probe set. The DNA molecules may be genomic DNA, protein nucleic acid (PNA), cDNA or synthetic oligonucleotides. Following the procedures taught herein, one can identify sequences of interest for analyzing gene expression in leukocytes. Such sequences may be predictive of a disease state.

Diagnostic oligonucleotides of the invention

The invention relates to diagnostic nucleotide set(s) comprising members of the leukocyte candidate library listed in Table 2, Table 3 and in the Sequence Listing, for which a correlation exists between the health status of an individual, and the individual's expression of RNA or protein products corresponding to the nucleotide sequence. In some instances, only one oligonucleotide is necessary for such detection. Members of a diagnostic oligonucleotide set may be identified by any means capable of detecting expression of RNA or protein products, including but not limited to differential expression screening, PCR, RT-PCR, SAGE analysis, high-throughput sequencing, microarrays, liquid or other arrays, protein-based methods (e.g., western blotting, proteomics, and other methods described herein), and data mining methods, as further described herein.

In one embodiment, a diagnostic oligonucleotide set comprises at least two oligonucleotide sequences listed in Table 2 or Table 3 or the Sequence Listing which are differentially expressed in leukocytes in an individual with at least one disease criterion for at least one leukocyte-implicated disease relative to the expression in individual without the at least one disease criterion, wherein expression of the two or more nucleotide sequences is correlated with at least one disease criterion, as described below. In another embodiment, a diagnostic nucleotide set comprises

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at least one oligonucleotide having an oligonucleotide sequence listed in Table 2 or 3 or the Sequence Listing which is differentially expressed, and further wherein the differential expression/correlation has not previously been described. In some embodiments, the diagnostic nucleotide set is immobilized on an array.

The invention also provides diagnostic probe sets. It is understood that a probe includes any reagent capable of specifically identifying a nucleotide sequence of the diagnostic nucleotide set, including but not limited to a DNA, a RNA, cDNA, synthetic oligonucleotide, partial or full-length nucleic acid sequences. In addition, the probe may identify the protein product of a diagnostic nucleotide sequence, including, for example, antibodies and other affinity reagents. It is also understood that each probe can correspond to one gene, or multiple probes can correspond to one gene, or both, or one probe can correspond to more than one gene.

Homologs and variants of the disclosed nucleic acid molecules may be used in the present invention. Homologs and variants of these nucleic acid molecules will possess a relatively high degree of sequence identity when aligned using standard methods. The sequences encompassed by the invention have at least 40-50, 50-60, 70-80, 80-85, 85-90, 90-95 or 95-100% sequence identity to the sequences disclosed herein.

It is understood that for expression profiling, variations in the disclosed sequences will still permit detection of gene expression. The degree of sequence identity required to detect gene expression varies depending on the length of the oligomer. For a 60 mer, 6-8 random mutations or 6-8 random deletions in a 60 mer do not affect gene expression detection. Hughes, TR, et al, "Expression profiling using microarrays fabricated by an ink-jet oligonucleotide synthesizer. *Nature Biotechnology*, 19:343-347(2001). As the length of the DNA sequence is increased, the number of mutations or deletions permitted while still allowing gene expression detection is increased.

As will be appreciated by those skilled in the art, the sequences of the present invention may contain sequencing errors. That is, there may be incorrect nucleotides, frameshifts, unknown nucleotides, or other types of sequencing errors in any of the sequences; however, the correct sequences will fall within the homology and stringency definitions herein.

The minimum length of an oligonucleotide probe necessary for specific hybridization in the human genome can be estimated using two approaches. The first method uses a statistical argument that the probe will be unique in the human genome by chance. Briefly, the number of independent perfect matches (P_o) expected for an oligonucleotide of length L in a genome of complexity C can be calculated from the equation (Laird CD, Chromosoma 32:378 (1971)):

$$P_o = (1/4)^L * 2C$$

In the case of mammalian genomes, $2C = \sim 3.6 \times 10^9$, and an oligonucleotide of 14-15 nucleotides is expected to be represented only once in the genome. However, the distribution of nucleotides in the coding sequence of mammalian genomes is nonrandom (Lathé, R. J. Mol. Biol. 183:1 (1985) and longer oligonucleotides may be preferred in order to increase the specificity of hybridization. In practical terms, this works out to probes that are 19-40 nucleotides long (Sambrook J et al., *infra*). The second method for estimating the length of a specific probe is to use a probe long enough to hybridize under the chosen conditions and use a computer to search for that sequence or close matches to the sequence in the human genome and choose a unique match. Probe sequences are chosen based on the desired hybridization properties as described in Chapter 11 of Sambrook et al, *infra*. The PRIMER3 program is useful for designing these probes (S. Rozen and H. Skaletsky 1996,1997; Primer3 code available at http://www-genome.wi.mit.edu/genome_software/other/primer3.html). The sequences of these probes are then compared pair wise against a database of the human genome sequences using a program such as BLAST or MEGABLAST (Madden, T.L et al.(1996) Meth. Enzymol. 266:131-141). Since most of the human genome is now contained in the database, the number of matches will be determined. Probe sequences are chosen that are unique to the desired target sequence.

In some embodiments, a diagnostic probe set is immobilized on an array. The array is optionally comprises one or more of: a chip array, a plate array, a bead array, a pin array, a membrane array, a solid surface array, a liquid array, an oligonucleotide array, a polynucleotide array or a cDNA array, a microtiter plate, a pin array, a bead array, a membrane or a chip.

In some embodiments, the leukocyte-implicated disease is selected from the diseases listed in Table 1. In other embodiments, the disease is atherosclerosis or

cardiac allograft rejection. In other embodiments, the disease is congestive heart failure, angina, myocardial infarction, systemic lupus erythematosus (SLE) and rheumatoid arthritis.

General Molecular Biology References

In the context of the invention, nucleic acids and/or proteins are manipulated according to well known molecular biology techniques. Detailed protocols for numerous such procedures are described in, e.g., in Ausubel et al. Current Protocols in Molecular Biology (supplemented through 2000) John Wiley & Sons, New York ("Ausubel"); Sambrook et al. Molecular Cloning - A Laboratory Manual (2nd Ed.), Vol. 1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989 ("Sambrook"), and Berger and Kimmel Guide to Molecular Cloning Techniques, Methods in Enzymology volume 152 Academic Press, Inc., San Diego, CA ("Berger").

In addition to the above references, protocols for in vitro amplification techniques, such as the polymerase chain reaction (PCR), the ligase chain reaction (LCR), Q-replicase amplification, and other RNA polymerase mediated techniques (e.g., NASBA), useful e.g., for amplifying cDNA probes of the invention, are found in Mullis et al. (1987) U.S. Patent No. 4,683,202; PCR Protocols A Guide to Methods and Applications (Innis et al. eds) Academic Press Inc. San Diego, CA (1990) ("Innis"); Arnheim and Levinson (1990) C&EN 36; The Journal Of NIH Research (1991) 3:81; Kwoh et al. (1989) Proc Natl Acad Sci USA 86, 1173; Guatelli et al. (1990) Proc Natl Acad Sci USA 87:1874; Lomell et al. (1989) J Clin Chem 35:1826; Landegren et al. (1988) Science 241:1077; Van Brunt (1990) Biotechnology 8:291; Wu and Wallace (1989) Gene 4: 560; Barringer et al. (1990) Gene 89:117, and Sooknanan and Malek (1995) Biotechnology 13:563. Additional methods, useful for cloning nucleic acids in the context of the present invention, include Wallace et al. U.S. Pat. No. 5,426,039. Improved methods of amplifying large nucleic acids by PCR are summarized in Cheng et al. (1994) Nature 369:684 and the references therein.

Certain polynucleotides of the invention, e.g., oligonucleotides can be synthesized utilizing various solid-phase strategies involving mononucleotide- and/or trinucleotide-based phosphoramidite coupling chemistry. For example, nucleic acid sequences can be synthesized by the sequential addition of activated monomers and/or

In lieu of synthesizing the desired sequences, essentially any nucleic acid can be custom ordered from any of a variety of commercial sources, such as The Midland Certified Reagent Company (mcrco@oligos.com), The Great American Gene Company (www.genco.com), ExpressGen, Inc. (www.expressgen.com), Operon Technologies, Inc. (www.operon.com), and many others.

Identification of diagnostic nucleotide sets

Libraries of candidates that are differentially expressed in leukocytes are substrates for the identification and evaluation of diagnostic oligonucleotide sets and disease specific target nucleotide sequences.

In addition to their widespread involvement and/or implication in numerous disease related processes, leukocytes are particularly attractive substrates for clinical and experimental evaluation for a variety of reasons. Most importantly, they are

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readily accessible at low cost from essentially every potential subject. Collection is minimally invasive and associated with little pain, disability or recovery time. Collection can be performed by minimally trained personnel (e.g., phlebotomists, medical technicians, etc.) in a variety of clinical and non-clinical settings without significant technological expenditure. Additionally, leukocytes are renewable, and thus available at multiple time points for a single subject.

Assembly of candidate libraries

At least two conceptually distinct approaches to the assembly of candidate libraries exist. Either, or both, or other, approaches can be favorably employed. The method of assembling, or identifying, candidate libraries is secondary to the criteria utilized for selecting appropriate library members. Most importantly, library members are assembled based on differential expression of RNA or protein products in leukocyte populations. More specifically, candidate nucleotide sequences are induced or suppressed, or expressed at increased or decreased levels in leukocytes from a subject with one or more disease or disease state (a disease criterion) relative to leukocytes from a subject lacking the specified disease criterion. Alternatively, or in addition, library members can be assembled from among nucleotide sequences that are differentially expressed in activated or resting leukocytes relative to other cell types.

Firstly, publication and sequence databases can be "mined" using a variety of search strategies, including, e.g., a variety of genomics and proteomics approaches. For example, currently available scientific and medical publication databases such as Medline, Current Contents, OMIM (online Mendelian inheritance in man) various Biological and Chemical Abstracts, Journal indexes, and the like can be searched using term or key-word searches, or by author, title, or other relevant search parameters. Many such databases are publicly available, and one of skill is well versed in strategies and procedures for identifying publications and their contents, e.g., genes, other nucleotide sequences, descriptions, indications, expression pattern, etc. Numerous databases are available through the internet for free or by subscription, *see*, e.g., <http://www.ncbi.nlm.nih.gov/PubMed/>; <http://www3.infotrieve.com/>; <http://www.isinet.com/>; <http://www.sciencemag.org/>. Additional or alternative publication or citation databases are also available that provide identical or similar types of information, any of which are favorably employed in the context of the invention. These databases can be searched for publications describing differential

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gene expression in leukocytes between patient with and without diseases or conditions listed in Table 1. We identified the nucleotide sequences listed in Table 2 and some of the sequences listed in Table 8 (Example 20), using data mining methods.

Alternatively, a variety of publicly available and proprietary sequence databases (including GenBank, dbEST, UniGene, and TIGR and SAGE databases) including sequences corresponding to expressed nucleotide sequences, such as expressed sequence tags (ESTs) are available. For example, Genbank™ (<http://www.ncbi.nlm.nih.gov/Genbank/>) among others can be readily accessed and searched via the internet. These and other sequence and clone database resources are currently available; however, any number of additional or alternative databases comprising nucleotide sequence sequences, EST sequences, clone repositories, PCR primer sequences, and the like corresponding to individual nucleotide sequence sequences are also suitable for the purposes of the invention. Sequences from nucleotide sequences can be identified that are only found in libraries derived from leukocytes or sub-populations of leukocytes, for example see Table 2.

Alternatively, the representation, or relative frequency, of a nucleotide sequence may be determined in a leukocyte-derived nucleic acid library and compared to the representation of the sequence in non-leukocyte derived libraries. The representation of a nucleotide sequence correlates with the relative expression level of the nucleotide sequence in leukocytes and non-leukocytes. An oligonucleotide sequence which has increased or decreased representation in a leukocyte-derived nucleic acid library relative to a non-leukocyte-derived libraries is a candidate for a leukocyte-specific gene.

Nucleotide sequences identified as having specificity to activated or resting leukocytes or to leukocytes from patients or patient samples with a variety of disease types can be isolated for use in a candidate library for leukocyte expression profiling through a variety of mechanisms. These include, but are not limited to, the amplification of the nucleotide sequence from RNA or DNA using nucleotide sequence specific primers for PCR or RT-PCR, isolation of the nucleotide sequence using conventional cloning methods, the purchase of an IMAGE consortium cDNA clone (EST) with complimentary sequence or from the same expressed nucleotide sequence, design of oligonucleotides, preparation of synthetic nucleic acid sequence, or any other nucleic-acid based method. In addition, the protein product of the

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nucleotide sequence can be isolated or prepared, and represented in a candidate library, using standard methods in the art, as described further below.

While the above discussion related primarily to “genomics” approaches, it is appreciated that numerous, analogous “proteomics” approaches are suitable to the present invention. For example, a differentially expressed protein product can, for example, be detected using western analysis, two-dimensional gel analysis, chromatographic separation, mass spectrometric detection, protein-fusion reporter constructs, colorimetric assays, binding to a protein array, or by characterization of polysomal mRNA. The protein is further characterized and the nucleotide sequence encoding the protein is identified using standard techniques, e.g. by screening a cDNA library using a probe based on protein sequence information.

The second approach involves the construction of a differential expression library by any of a variety of means. Any one or more of differential screening, differential display or subtractive hybridization procedures, or other techniques that preferentially identify, isolate or amplify differentially expressed nucleotide sequences can be employed to produce a library of differentially expressed candidate nucleotide sequences, a subset of such a library, a partial library, or the like. Such methods are well known in the art. For example, peripheral blood leukocytes, (i.e., a mixed population including lymphocytes, monocytes and neutrophils), from multiple donor samples are pooled to prevent bias due to a single-donor's unique genotype. The pooled leukocytes are cultured in standard medium and stimulated with individual cytokines or growth factors e.g., with IL-2, IL-1, MCP1, TNF α , and/or IL8 according to well known procedures (*see*, e.g., Tough et al. (1999) ; Winston et al. (1999); Hansson et al. (1989)). Typically, leukocytes are recovered from Buffy coat preparations produced by centrifugation of whole blood. Alternatively, mononuclear cells (monocytes and lymphocytes) can be obtained by density gradient centrifugation of whole blood, or specific cell types (such as a T lymphocyte) can be isolated using affinity reagents to cell specific surface markers. Leukocytes may also be stimulated by incubation with ionomycin, and phorbol myristate acetate (PMA). This stimulation protocol is intended to non-specifically mimic “activation” of numerous pathways due to variety of disease conditions rather than to simulate any single disease condition or paradigm.

Using well known subtractive hybridization procedures (as described in, e.g., US Patent Numbers 5,958,738; 5,589,339; 5,827,658; 5,712,127; 5,643,761) a library

is produced that is enriched for RNA species (messages) that are differentially expressed between test and control leukocyte populations. In some embodiments, the test population of leukocytes are simply stimulated as described above to emulate non-specific activation events, while in other embodiments the test population can be selected from subjects (or patients) with a specified disease or class of diseases. Typically, the control leukocyte population lacks the defining test condition, e.g., stimulation, disease state, diagnosis, genotype, etc. Alternatively, the total RNA from control and test leukocyte populations are prepared by established techniques, treated with DNaseI, and selected for messenger RNA with an intact 3' end (i.e., polyA(+)) messenger RNA) e.g., using commercially available kits according to the manufacturer's instructions e.g. Clontech. Double stranded cDNA is synthesized utilizing reverse transcriptase. Double stranded cDNA is then cut with a first restriction enzyme (e.g., *NlaIII*, that cuts at the recognition site: CATG, and cuts the cDNA sequence at approximately 256 bp intervals) that cuts the cDNA molecules into conveniently sized fragments.

The cDNAs prepared from the test population of leukocytes are divided into (typically 2) "tester" pools, while cDNAs prepared from the control population of leukocytes are designated the "driver" pool. Typically, pooled populations of cells from multiple individual donors are utilized and in the case of stimulated versus unstimulated cells, the corresponding tester and driver pools for any single subtraction reaction are derived from the same donor pool.

A unique double-stranded adapter is ligated to each of the tester cDNA populations using unphosphorylated primers so that only the sense strand is covalently linked to the adapter. An initial hybridization is performed consisting of each of the tester pools of cDNA (each with its corresponding adapter) and an excess of the driver cDNA. Typically, an excess of about 10-100 fold driver relative to tester is employed, although significantly lower or higher ratios can be empirically determined to provide more favorable results. The initial hybridization results in an initial normalization of the cDNAs such that high and low abundance messages become more equally represented following hybridization due to a failure of driver/tester hybrids to amplify.

A second hybridization involves pooling un-hybridized sequences from initial hybridizations together with the addition of supplemental driver cDNA. In this step, the expressed sequences enriched in the two tester pools following the initial

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Either of the above approaches, or both in combination, or indeed, any procedure, which permits the assembly of a collection of nucleotide sequences that are expressed in leukocytes, is favorably employed to produce the libraries of candidates useful for the identification of diagnostic nucleotide sets and disease specific target nucleotides of the invention. Additionally, any method that permits the assembly of a collection of nucleotides that are expressed in leukocytes and preferentially associated with one or more disease or condition, whether or not the nucleotide sequences are differentially expressed, is favorably employed in the context of the invention. Typically, libraries of about 2,000-10,000 members are produced (although libraries in excess of 10,000 are not uncommon). Following additional evaluation procedures, as described below, the proportion of unique clones in the candidate library can approximate 100%.

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A candidate oligonucleotide sequence may be represented in a candidate library by a full-length or partial nucleic acid sequence, deoxyribonucleic acid (DNA) sequence, cDNA sequence, RNA sequence, synthetic oligonucleotides, etc. The nucleic acid sequence can be at least 19 nucleotides in length, at least 25 nucleotides, at least 40 nucleotides, at least 100 nucleotides, or larger. Alternatively, the protein product of a candidate nucleotide sequence may be represented in a candidate library using standard methods, as further described below.

Characterization of candidate oligonucleotide sequences

The sequence of individual members (e.g., clones, partial sequence listing in a database such as an EST, etc.) of the candidate oligonucleotide libraries is then determined by conventional sequencing methods well known in the art, e.g., by the dideoxy-chain termination method of Sanger et al. (1977) Proc Natl Acad Sci USA 74:5463-7; by chemical procedures, e.g., Maxam and Gilbert (1977) Proc Natl Acad Sci USA 74:560-4; or by polymerase chain reaction cycle sequencing methods, e.g., Olsen and Eckstein (1989) Nuc Acid Res 17:9613-20, DNA chip based sequencing techniques or variations, including automated variations (e.g., as described in Hunkapiller et al. (1991) Science 254:59-67; Pease et al. (1994) Proc Natl Acad Sci USA 91:5022-6), thereof. Numerous kits for performing the above procedures are commercially available and well known to those of skill in the art. Character strings corresponding to the resulting nucleotide sequences are then recorded (i.e., stored) in a database. Most commonly the character strings are recorded on a computer readable medium for processing by a computational device.

Generally, to facilitate subsequent analysis, a custom algorithm is employed to query existing databases in an ongoing fashion, to determine the identity, expression pattern and potential function of the particular members of a candidate library. The sequence is first processed, by removing low quality sequence. Next the vector sequences are identified and removed and sequence repeats are identified and masked. The remaining sequence is then used in a Blast algorithm against multiple publicly available, and/or proprietary databases, e.g., NCBI nucleotide, EST and protein databases, Unigene, and Human Genome Sequence. Sequences are also compared to all previously sequenced members of the candidate libraries to detect redundancy.

In some cases, sequences are of high quality, but do not match any sequence in the NCBI nr, human EST or Unigene databases. In this case the sequence is queried against the human genomic sequence. If a single chromosomal site is matched with a

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high degree of confidence, that region of genomic DNA is identified and subjected to further analysis with a gene prediction program such as GRAIL. This analysis may lead to the identification of a new gene in the genomic sequence. This sequence can then be translated to identify the protein sequence that is encoded and that sequence can be further analyzed using tools such as Pfam, Blast P, or other protein structure prediction programs, as illustrated in Table 7. Typically, the above analysis is directed towards the identification of putative coding regions, e.g., previously unidentified open reading frames, confirming the presence of known coding sequences, and determining structural motifs or sequence similarities of the predicted protein (i.e., the conceptual translation product) in relation to known sequences. In addition, it has become increasingly possible to assemble "virtual cDNAs" containing large portions of coding region, simply through the assembly of available expressed sequence tags (ESTs). In turn, these extended nucleic acid and amino acid sequences allow the rapid expansion of substrate sequences for homology searches and structural and functional motif characterization. The results of these analysis permits the categorization of sequences according to structural characteristics, e.g., as structural proteins, proteins involved in signal transduction, cell surface or secreted proteins etc.

It is understood that full-length nucleotide sequences may also be identified using conventional methods, for example, library screening, RT-PCR, chromosome walking, etc., as described in *Sambrook and Ausebel, infra*.

Candidate nucleotide library of the invention

We identified members of a candidate nucleotide library that are differentially expressed in activated leukocytes and resting leukocytes. Accordingly, the invention provides the candidate leukocyte nucleotide library comprising the nucleotide sequences listed in Table 2, Table 3, and in the sequence listing. In another embodiment, the invention provides a candidate library comprising at least two nucleotide sequences listed in Table 2, Table 3, and the sequence listing. In another embodiment, the at least two nucleotide sequence are at least 19 nucleotides in length, at least 35 nucleotides, at least 40 nucleotides or at least 100 nucleotides. In some embodiments, the nucleotide sequences comprises deoxyribonucleic acid (DNA) sequence, ribonucleic acid (RNA) sequence, synthetic oligonucleotide sequence, or genomic DNA sequence. It is understood that the nucleotide sequences may each

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correspond to one gene, or that several nucleotide sequences may correspond to one gene, or both.

The invention also provides probes to the candidate nucleotide library. In one embodiment of the invention, the probes comprise at least two nucleotide sequences listed in Table 2, Table 3, or the sequence listing which are differentially expressed in leukocytes in an individual with a least one disease criterion for at least one leukocyte-related disease and in leukocytes in an individual without the at least one disease criterion, wherein expression of the two or more nucleotide sequences is correlated with at least one disease criterion. It is understood that a probe may detect either the RNA expression or protein product expression of the candidate nucleotide library. Alternatively, or in addition, a probe can detect a genotype associated with a candidate nucleotide sequence, as further described below. In another embodiment, the probes for the candidate nucleotide library are immobilized on an array.

The candidate nucleotide library of the invention is useful in identifying diagnostic nucleotide sets of the invention, as described below. The candidate nucleotide sequences may be further characterized, and may be identified as a disease target nucleotide sequence and/or a novel nucleotide sequence, as described below. The candidate nucleotide sequences may also be suitable for use as imaging reagents, as described below.

Generation of Expression Patterns

RNA, DNA or protein sample procurement

Following identification or assembly of a library of differentially expressed candidate nucleotide sequences, leukocyte expression profiles corresponding to multiple members of the candidate library are obtained. Leukocyte samples from one or more subjects are obtained by standard methods. Most typically, these methods involve trans-cutaneous venous sampling of peripheral blood. While sampling of circulating leukocytes from whole blood from the peripheral vasculature is generally the simplest, least invasive, and lowest cost alternative, it will be appreciated that numerous alternative sampling procedures exist, and are favorably employed in some circumstances. No pertinent distinction exists, in fact, between leukocytes sampled from the peripheral vasculature, and those obtained, e.g., from a central line, from a central artery, or indeed from a cardiac catheter, or during a surgical procedure which accesses the central vasculature. In addition, other body fluids and tissues that are, at

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least in part, composed of leukocytes, are also desirable leukocyte samples. For example, fluid samples obtained from the lung during bronchoscopy may be rich in leukocytes, and amenable to expression profiling in the context of the invention, e.g., for the diagnosis, prognosis, or monitoring of lung transplant rejection, inflammatory lung diseases or infectious lung disease. Fluid samples from other tissues, e.g., obtained by endoscopy of the colon, sinuses, esophagus, stomach, small bowel, pancreatic duct, biliary tree, bladder, ureter, vagina, cervix or uterus, etc., are also suitable. Samples may also be obtained other sources containing leukocytes, e.g., from urine, bile, cerebrospinal fluid, feces, gastric or intestinal secretions, semen, or solid organ or joint biopsies.

Most frequently, mixed populations of leukocytes, such as are found in whole blood are utilized in the methods of the present invention. A crude separation, e.g., of mixed leukocytes from red blood cells, and/or concentration, e.g., over a sucrose, percoll or ficoll gradient, or by other methods known in the art, can be employed to facilitate the recovery of RNA or protein expression products at sufficient concentrations, and to reduce non-specific background. In some instances, it can be desirable to purify sub-populations of leukocytes, and methods for doing so, such as density or affinity gradients, flow cytometry, fluorescence Activated Cell Sorting (FACS), immuno-magnetic separation, "panning," and the like, are described in the available literature and below.

Obtaining DNA, RNA and protein samples for expression profiling

Expression patterns can be evaluated at the level of DNA, or RNA or protein products. For example, a variety of techniques are available for the isolation of RNA from whole blood. Any technique that allows isolation of mRNA from cells (in the presence or absence of rRNA and tRNA) can be utilized. In brief, one method that allows reliable isolation of total RNA suitable for subsequent gene expression analysis, is described as follows. Peripheral blood (either venous or arterial) is drawn from a subject, into one or more sterile, endotoxin free, tubes containing an anticoagulant (e.g., EDTA, citrate, heparin, etc.). Typically, the sample is divided into at least two portions. One portion, e.g., of 5-8 ml of whole blood is frozen and stored for future analysis, e.g., of DNA or protein. A second portion, e.g., of approximately 8 ml whole blood is processed for isolation of total RNA by any of a

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variety of techniques as described in, e.g, Sambrook, Ausubel, below, as well as U.S. Patent Numbers: 5,728,822 and 4,843,155.

Typically, a subject sample of mononuclear leukocytes obtained from about 8 ml of whole blood, a quantity readily available from an adult human subject under most circumstances, yields 5-20 μ g of total RNA. This amount is ample, e.g., for labeling and hybridization to at least two probe arrays. Labeled probes for analysis of expression patterns of nucleotides of the candidate libraries are prepared from the subject's sample of RNA using standard methods. In many cases, cDNA is synthesized from total RNA using a polyT primer and labeled, e.g., radioactive or fluorescent, nucleotides. The resulting labeled cDNA is then hybridized to probes corresponding to members of the candidate nucleotide library, and expression data is obtained for each nucleotide sequence in the library. RNA isolated from subject samples (e.g., peripheral blood leukocytes, or leukocytes obtained from other biological fluids and samples) is next used for analysis of expression patterns of nucleotides of the candidate libraries.

In some cases, however, the amount of RNA that is extracted from the leukocyte sample is limiting, and amplification of the RNA is desirable. Amplification may be accomplished by increasing the efficiency of probe labeling, or by amplifying the RNA sample prior to labeling. It is appreciated that care must be taken to select an amplification procedure that does not introduce any bias (with respect to gene expression levels) during the amplification process.

Several methods are available that increase the signal from limiting amounts of RNA, e.g. use of the Clontech (Glass Fluorescent Labeling Kit) or Stratagene (Fairplay Microarray Labeling Kit), or the Micromax kit (New England Nuclear, Inc.). Alternatively, cDNA is synthesized from RNA using a T7- polyT primer, in the absence of label, and DNA dendrimers from Genisphere (3DNA Submicro) are hybridized to the poly T sequence on the primer, or to a different "capture sequence" which is complementary to a fluorescently labeled sequence. Each 3DNA molecule has 250 fluorescent molecules and therefore can strongly label each cDNA.

Alternatively, the RNA sample is amplified prior to labeling. For example, linear amplification may be performed, as described in U.S. Patent No. 6,132,997. A T7-polyT primer is used to generate the cDNA copy of the RNA. A second DNA strand is then made to complete the substrate for amplification. The T7 promoter

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incorporated into the primer is used by a T7 polymerase to produce numerous antisense copies of the original RNA. Fluorescent dye labeled nucleotides are directly incorporated into the RNA. Alternatively, amino allyl labeled nucleotides are incorporated into the RNA, and then fluorescent dyes are chemically coupled to the amino allyl groups, as described in Hughes. Other exemplary methods for amplification are described below.

It is appreciated that the RNA isolated must contain RNA derived from leukocytes, but may also contain RNA from other cell types to a variable degree. Additionally, the isolated RNA may come from subsets of leukocytes, e.g. monocytes and/or T-lymphocytes, as described above. Such consideration of cell type used for the derivation of RNA depend on the method of expression profiling used.

DNA samples may be obtained for analysis of the presence of DNA mutations, single nucleotide polymorphisms (SNPs), or other polymorphisms. DNA is isolated using standard techniques, e.g. *Maniatus, supra*.

Expression of products of candidate nucleotides may also be assessed using proteomics. Protein(s) are detected in samples of patient serum or from leukocyte cellular protein. Serum is prepared by centrifugation of whole blood, using standard methods. Proteins present in the serum may have been produced from any of a variety of leukocytes and non-leukocyte cells, and include secreted proteins from leukocytes. Alternatively, leukocytes or a desired sub-population of leukocytes are prepared as described above. Cellular protein is prepared from leukocyte samples using methods well known in the art, e.g., Trizol (Invitrogen Life Technologies, cat # 15596108; Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* 162, 156; Simms, D., Cizdziel, P.E., and Chomczynski, P. (1993) *Focus* 15, 99; Chomczynski, P., Bowers-Finn, R., and Sabatini, L. (1987) *J. of NIH Res.* 6, 83; Chomczynski, P. (1993) *Bio/Techniques* 15, 532; Bracete, A.M., Fox, D.K., and Simms, D. (1998) *Focus* 20, 82; Sewall, A. and McRae, S. (1998) *Focus* 20, 36; *Anal Biochem* 1984 Apr;138(1):141-3, A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids; Wessel D, Flugge UI. (1984) *Anal Biochem.* 1984 Apr;138(1):141-143.

Obtaining expression patterns

Expression patterns, or profiles, of a plurality of nucleotides corresponding to members of the candidate library are then evaluated in one or more samples of leukocytes. Typically, the leukocytes are derived from patient peripheral blood

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samples, although, as indicated above, many other sample sources are also suitable. These expression patterns constitute a set of relative or absolute expression values for a some number of RNAs or protein products corresponding to the plurality of nucleotide sequences evaluated, which is referred to herein as the subject's "expression profile" for those nucleotide sequences. While expression patterns for as few as one independent member of the candidate library can be obtained, it is generally preferable to obtain expression patterns corresponding to a larger number of nucleotide sequences, e.g., about 2, about 5, about 10, about 20, about 50, about 100, about 200, about 500, or about 1000, or more. The expression pattern for each differentially expressed component member of the library provides a finite specificity and sensitivity with respect to predictive value, e.g., for diagnosis, prognosis, monitoring, and the like.

Clinical Studies, Data and Patient Groups

For the purpose of discussion, the term subject, or subject sample of leukocytes, refers to an individual regardless of health and/or disease status. A subject can be a patient, a study participant, a control subject, a screening subject, or any other class of individual from whom a leukocyte sample is obtained and assessed in the context of the invention. Accordingly, a subject can be diagnosed with a disease, can present with one or more symptom of a disease, or a predisposing factor, such as a family (genetic) or medical history (medical) factor, for a disease, or the like. Alternatively, a subject can be healthy with respect to any of the aforementioned factors or criteria. It will be appreciated that the term "healthy" as used herein, is relative to a specified disease, or disease factor, or disease criterion, as the term "healthy" cannot be defined to correspond to any absolute evaluation or status. Thus, an individual defined as healthy with reference to any specified disease or disease criterion, can in fact be diagnosed with any other one or more disease, or exhibit any other one or more disease criterion.

Furthermore, while the discussion of the invention focuses, and is exemplified using human sequences and samples, the invention is equally applicable, through construction or selection of appropriate candidate libraries, to non-human animals, such as laboratory animals, e.g., mice, rats, guinea pigs, rabbits; domesticated livestock, e.g., cows, horses, goats, sheep, chicken, etc.; and companion animals, e.g., dogs, cats, etc.

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Methods for obtaining expression data

Numerous methods for obtaining expression data are known, and any one or more of these techniques, singly or in combination, are suitable for determining expression profiles in the context of the present invention. For example, expression patterns can be evaluated by northern analysis, PCR, RT-PCR, Taq Man analysis, FRET detection, monitoring one or more molecular beacon, hybridization to an oligonucleotide array, hybridization to a cDNA array, hybridization to a polynucleotide array, hybridization to a liquid microarray, hybridization to a microelectric array, molecular beacons, cDNA sequencing, clone hybridization, cDNA fragment fingerprinting, serial analysis of gene expression (SAGE), subtractive hybridization, differential display and/or differential screening (*see, e.g.,* Lockhart and Winzeler (2000) Nature 405:827-836, and references cited therein).

For example, specific PCR primers are designed to a member(s) of a candidate nucleotide library. cDNA is prepared from subject sample RNA by reverse transcription from a poly-dT oligonucleotide primer, and subjected to PCR. Double stranded cDNA may be prepared using primers suitable for reverse transcription of the PCR product, followed by amplification of the cDNA using in vitro transcription. The product of in vitro transcription is a sense-RNA corresponding to the original member(s) of the candidate library. PCR product may be also be evaluated in a number of ways known in the art, including real-time assessment using detection of labeled primers, e.g. TaqMan or molecular beacon probes. Technology platforms suitable for analysis of PCR products include the ABI 7700, 5700, or 7000 Sequence Detection Systems (Applied Biosystems, Foster City, CA), the MJ Research Opticon (MJ Research, Waltham, MA), the Roche Light Cycler (Roche Diagnostics, Indianapolis, IN), the Stratagene MX4000 (Stratagene, La Jolla, CA), and the Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA). Alternatively, molecular beacons are used to detect presence of a nucleic acid sequence in an unamplified RNA or cDNA sample, or following amplification of the sequence using any method, e.g. IVT (In Vitro transcription) or NASBA (nucleic acid sequence based amplification). Molecular beacons are designed with sequences complementary to member(s) of a candidate nucleotide library, and are linked to fluorescent labels. Each probe has a different fluorescent label with non-overlapping emission wavelengths. For example,

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expression of ten genes may be assessed using ten different sequence-specific molecular beacons.

Alternatively, or in addition, molecular beacons are used to assess expression of multiple nucleotide sequences at once. Molecular beacons with sequence complimentary to the members of a diagnostic nucleotide set are designed and linked to fluorescent labels. Each fluorescent label used must have a non-overlapping emission wavelength. For example, 10 nucleotide sequences can be assessed by hybridizing 10 sequence specific molecular beacons (each labeled with a different fluorescent molecule) to an amplified or un-amplified RNA or cDNA sample. Such an assay bypasses the need for sample labeling procedures.

Alternatively, or in addition bead arrays can be used to assess expression of multiple sequences at once. See, e.g., LabMAP 100, Luminex Corp, Austin, Texas). Alternatively, or in addition electric arrays are used to assess expression of multiple sequences, as exemplified by the e-Sensor technology of Motorola (Chicago, Ill.) or Nanochip technology of Nanogen (San Diego, CA.)

Of course, the particular method elected will be dependent on such factors as quantity of RNA recovered, practitioner preference, available reagents and equipment, detectors, and the like. Typically, however, the elected method(s) will be appropriate for processing the number of samples and probes of interest. Methods for high-throughput expression analysis are discussed below.

Alternatively, expression at the level of protein products of gene expression is performed. For example, protein expression, in a sample of leukocytes, can be evaluated by one or more method selected from among: western analysis, two-dimensional gel analysis, chromatographic separation, mass spectrometric detection, protein-fusion reporter constructs, colorimetric assays, binding to a protein array and characterization of polysomal mRNA. One particularly favorable approach involves binding of labeled protein expression products to an array of antibodies specific for members of the candidate library. Methods for producing and evaluating antibodies are widespread in the art, *see*, e.g., Coligan, *supra*; and Harlow and Lane (1989) Antibodies: A Laboratory Manual, Cold Spring Harbor Press, NY ("Harlow and Lane"). Additional details regarding a variety of immunological and immunoassay procedures adaptable to the present invention by selection of antibody reagents specific for the products of candidate nucleotide sequences can be found in, e.g., Stites and Terr (eds.)(1991) Basic and Clinical Immunology, 7th ed., and Paul, *supra*.

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Another approach uses systems for performing desorption spectrometry. Commercially available systems, e.g., from Ciphergen Biosystems, Inc. (Fremont, CA) are particularly well suited to quantitative analysis of protein expression. Indeed, Protein Chip® arrays (*see*, e.g., <http://www.ciphergen.com/>) used in desorption spectrometry approaches provide arrays for detection of protein expression. Alternatively, affinity reagents, e.g., antibodies, small molecules, etc.) are developed that recognize epitopes of the protein product. Affinity assays are used in protein array assays, e.g. to detect the presence or absence of particular proteins. Alternatively, affinity reagents are used to detect expression using the methods described above. In the case of a protein that is expressed on the cell surface of leukocytes, labeled affinity reagents are bound to populations of leukocytes, and leukocytes expressing the protein are identified and counted using fluorescent activated cell sorting (FACS).

It is appreciated that the methods of expression evaluation discussed herein, although discussed in the context of discovery of diagnostic nucleotide sets, are equally applicable for expression evaluation when using diagnostic nucleotide sets for, e.g. diagnosis of diseases, as further discussed below.

High Throughput Expression Assays

A number of suitable high throughput formats exist for evaluating gene expression. Typically, the term high throughput refers to a format that performs at least about 100 assays, or at least about 500 assays, or at least about 1000 assays, or at least about 5000 assays, or at least about 10,000 assays, or more per day. When enumerating assays, either the number of samples or the number of candidate nucleotide sequences evaluated can be considered. For example, a northern analysis of, e.g., about 100 samples performed in a gridded array, e.g., a dot blot, using a single probe corresponding to a candidate nucleotide sequence can be considered a high throughput assay. More typically, however, such an assay is performed as a series of duplicate blots, each evaluated with a distinct probe corresponding to a different member of the candidate library. Alternatively, methods that simultaneously evaluate expression of about 100 or more candidate nucleotide sequences in one or more samples, or in multiple samples, are considered high throughput.

Numerous technological platforms for performing high throughput expression analysis are known. Generally, such methods involve a logical or physical array of

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either the subject samples, or the candidate library, or both. Common array formats include both liquid and solid phase arrays. For example, assays employing liquid phase arrays, e.g., for hybridization of nucleic acids, binding of antibodies or other receptors to ligand, etc., can be performed in multiwell, or microtiter, plates. Microtiter plates with 96, 384 or 1536 wells are widely available, and even higher numbers of wells, e.g., 3456 and 9600 can be used. In general, the choice of microtiter plates is determined by the methods and equipment, e.g., robotic handling and loading systems, used for sample preparation and analysis. Exemplary systems include, e.g., the ORCA™ system from Beckman-Coulter, Inc. (Fullerton, CA) and the Zymate systems from Zymark Corporation (Hopkinton, MA).

Alternatively, a variety of solid phase arrays can favorably be employed in to determine expression patterns in the context of the invention. Exemplary formats include membrane or filter arrays (e.g., nitrocellulose, nylon), pin arrays, and bead arrays (e.g., in a liquid "slurry"). Typically, probes corresponding to nucleic acid or protein reagents that specifically interact with (e.g., hybridize to or bind to) an expression product corresponding to a member of the candidate library are immobilized, for example by direct or indirect cross-linking, to the solid support. Essentially any solid support capable of withstanding the reagents and conditions necessary for performing the particular expression assay can be utilized. For example, functionalized glass, silicon, silicon dioxide, modified silicon, any of a variety of polymers, such as (poly)tetrafluoroethylene, (poly)vinylidenedifluoride, polystyrene, polycarbonate, or combinations thereof can all serve as the substrate for a solid phase array.

In a preferred embodiment, the array is a "chip" composed, e.g., of one of the above specified materials. Polynucleotide probes, e.g., RNA or DNA, such as cDNA, synthetic oligonucleotides, and the like, or binding proteins such as antibodies, that specifically interact with expression products of individual components of the candidate library are affixed to the chip in a logically ordered manner, i.e., in an array. In addition, any molecule with a specific affinity for either the sense or anti-sense sequence of the marker nucleotide sequence (depending on the design of the sample labeling), can be fixed to the array surface without loss of specific affinity for the marker and can be obtained and produced for array production, for example, proteins that specifically recognize the specific nucleic acid sequence of the marker,

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ribozymes, peptide nucleic acids (PNA), or other chemicals or molecules with specific affinity.

Detailed discussion of methods for linking nucleic acids and proteins to a chip substrate, are found in, e.g., US Patent No. 5,143,854 "LARGE SCALE PHOTOLITHOGRAPHIC SOLID PHASE SYNTHESIS OF POLYPEPTIDES AND RECEPTOR BINDING SCREENING THEREOF" to Pirrung et al., issued, September 1, 1992; US Patent No. 5,837,832 "ARRAYS OF NUCLEIC ACID PROBES ON BIOLOGICAL CHIPS" to Chee et al., issued November 17, 1998; US Patent No. 6,087,112 "ARRAYS WITH MODIFIED OLIGONUCLEOTIDE AND POLYNUCLEOTIDE COMPOSITIONS" to Dale, issued July 11, 2000; US Patent No. 5,215,882 "METHOD OF IMMOBILIZING NUCLEIC ACID ON A SOLID SUBSTRATE FOR USE IN NUCLEIC ACID HYBRIDIZATION ASSAYS" to Bahl et al., issued June 1, 1993; US Patent No. 5,707,807 "MOLECULAR INDEXING FOR EXPRESSED GENE ANALYSIS" to Kato, issued January 13, 1998; US Patent No. 5,807,522 "METHODS FOR FABRICATING MICROARRAYS OF BIOLOGICAL SAMPLES" to Brown et al., issued September 15, 1998; US Patent No. 5,958,342 "JET DROPLET DEVICE" to Gamble et al., issued Sept. 28, 1999; US Patent 5,994,076 "METHODS OF ASSAYING DIFFERENTIAL EXPRESSION" to Chenchik et al., issued Nov. 30, 1999; US Patent No. 6,004,755 "QUANTITATIVE MICROARRAY HYBRIDIZATION ASSAYS" to Wang, issued Dec. 21, 1999; US Patent No. 6,048,695 "CHEMICALLY MODIFIED NUCLEIC ACIDS AND METHOD FOR COUPLING NUCLEIC ACIDS TO SOLID SUPPORT" to Bradley et al., issued April 11, 2000; US Patent No. 6,060,240 "METHODS FOR MEASURING RELATIVE AMOUNTS OF NUCLEIC ACIDS IN A COMPLEX MIXTURE AND RETRIEVAL OF SPECIFIC SEQUENCES THEREFROM" to Kamb et al., issued May 9, 2000; US Patent No. 6,090,556 "METHOD FOR QUANTITATIVELY DETERMINING THE EXPRESSION OF A GENE" to Kato, issued July 18, 2000; and US Patent 6,040,138 "EXPRESSION MONITORING BY HYBRIDIZATION TO HIGH DENSITY OLIGONUCLEOTIDE ARRAYS" to Lockhart et al., issued March 21, 2000.

For example, cDNA inserts corresponding to candidate nucleotide sequences, in a standard TA cloning vector are amplified by a polymerase chain reaction for approximately 30-40 cycles. The amplified PCR products are then arrayed onto a glass support by any of a variety of well known techniques, e.g., the VSLIPS™

technology described in US Patent No. 5,143,854. RNA, or cDNA corresponding to RNA, isolated from a subject sample of leukocytes is labeled, e.g., with a fluorescent tag, and a solution containing the RNA (or cDNA) is incubated under conditions favorable for hybridization, with the "probe" chip. Following incubation, and washing to eliminate non-specific hybridization, the labeled nucleic acid bound to the chip is detected qualitatively or quantitatively, and the resulting expression profile for the corresponding candidate nucleotide sequences is recorded. It is appreciated that the probe used for diagnostic purposes may be identical to the probe used during diagnostic nucleotide sequence discovery and validation. Alternatively, the probe sequence may be different than the sequence used in diagnostic nucleotide sequence discovery and validation. Multiple cDNAs from a nucleotide sequence that are non-overlapping or partially overlapping may also be used.

In another approach, oligonucleotides corresponding to members of a candidate nucleotide library are synthesized and spotted onto an array. Alternatively, oligonucleotides are synthesized onto the array using methods known in the art, e.g. Hughes, et al. *supra*. The oligonucleotide is designed to be complementary to any portion of the candidate nucleotide sequence. In addition, in the context of expression analysis for, e.g. diagnostic use of diagnostic nucleotide sets, an oligonucleotide can be designed to exhibit particular hybridization characteristics, or to exhibit a particular specificity and/or sensitivity, as further described below.

Hybridization signal may be amplified using methods known in the art, and as described herein, for example use of the Clontech kit (Glass Fluorescent Labeling Kit), Stratagene kit (Fairplay Microarray Labeling Kit), the Micromax kit (New England Nuclear, Inc.), the Genisphere kit (3DNA Submicro), linear amplification, e.g. as described in U.S. Patent No. 6,132,997 or described in Hughes, TR, et al., *Nature Biotechnology*, 19:343-347 (2001) and/or Westin et al. *Nat Biotech.* 18:199-204.

Alternatively, fluorescently labeled cDNA are hybridized directly to the microarray using methods known in the art. For example, labeled cDNA are generated by reverse transcription using Cy3- and Cy5-conjugated deoxynucleotides, and the reaction products purified using standard methods. It is appreciated that the methods for signal amplification of expression data useful for identifying diagnostic nucleotide sets are also useful for amplification of expression data for diagnostic purposes.

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Microarray expression may be detected by scanning the microarray with a variety of laser or CCD-based scanners, and extracting features with numerous software packages, for example, Imagene (Biodiscovery), Feature Extraction (Agilent), Scanalyze (Eisen, M. 1999. SCANALYZE User Manual; Stanford Univ., Stanford, CA. Ver 2.32.), GenePix (Axon Instruments).

In another approach, hybridization to microelectric arrays is performed, e.g. as described in Umek et al (2001) J Mol Diagn. 3:74-84. An affinity probe, e.g. DNA, is deposited on a metal surface. The metal surface underlying each probe is connected to a metal wire and electrical signal detection system. Unlabelled RNA or cDNA is hybridized to the array, or alternatively, RNA or cDNA sample is amplified before hybridization, e.g. by PCR. Specific hybridization of sample RNA or cDNA results in generation of an electrical signal, which is transmitted to a detector. See Westin (2000) Nat Biotech. 18:199-204 (describing anchored multiplex amplification of a microelectronic chip array); Edman (1997) NAR 25:4907-14; Vignali (2000) J Immunol Methods 243:243-55.

In another approach, a microfluidics chip is used for RNA sample preparation and analysis. This approach increases efficiency because sample preparation and analysis are streamlined. Briefly, microfluidics may be used to sort specific leukocyte sub-populations prior to RNA preparation and analysis. Microfluidics chips are also useful for, e.g., RNA preparation, and reactions involving RNA (reverse transcription, RT-PCR). Briefly, a small volume of whole, anti-coagulated blood is loaded onto a microfluidics chip, for example chips available from Caliper (Mountain View, CA) or Nanogen (San Diego, CA.) A microfluidics chip may contain channels and reservoirs in which cells are moved and reactions are performed. Mechanical, electrical, magnetic, gravitational, centrifugal or other forces are used to move the cells and to expose them to reagents. For example, cells of whole blood are moved into a chamber containing hypotonic saline, which results in selective lysis of red blood cells after a 20-minute incubation. Next, the remaining cells (leukocytes) are moved into a wash chamber and finally, moved into a chamber containing a lysis buffer such as guanidine isothiocyanate. The leukocyte cell lysate is further processed for RNA isolation in the chip, or is then removed for further processing, for example, RNA extraction by standard methods. Alternatively, the microfluidics chip is a circular disk containing ficoll or another density reagent. The blood sample is injected into the center of the disc, the disc is rotated at a speed that generates a

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centrifugal force appropriate for density gradient separation of mononuclear cells, and the separated mononuclear cells are then harvested for further analysis or processing.

It is understood that the methods of expression evaluation, above, although discussed in the context of discovery of diagnostic nucleotide sets, are also applicable for expression evaluation when using diagnostic nucleotide sets for, e.g. diagnosis of diseases, as further discussed below.

Evaluation of expression patterns

Expression patterns can be evaluated by qualitative and/or quantitative measures. Certain of the above described techniques for evaluating gene expression (as RNA or protein products) yield data that are predominantly qualitative in nature. That is, the methods detect differences in expression that classify expression into distinct modes without providing significant information regarding quantitative aspects of expression. For example, a technique can be described as a qualitative technique if it detects the presence or absence of expression of a candidate nucleotide sequence, i.e., an on/off pattern of expression. Alternatively, a qualitative technique measures the presence (and/or absence) of different alleles, or variants, of a gene product.

In contrast, some methods provide data that characterizes expression in a quantitative manner. That is, the methods relate expression on a numerical scale, e.g., a scale of 0-5, a scale of 1-10, a scale of + - +++, from grade 1 to grade 5, a grade from a to z, or the like. It will be understood that the numerical, and symbolic examples provided are arbitrary, and that any graduated scale (or any symbolic representation of a graduated scale) can be employed in the context of the present invention to describe quantitative differences in nucleotide sequence expression. Typically, such methods yield information corresponding to a relative increase or decrease in expression.

Any method that yields either quantitative or qualitative expression data is suitable for evaluating expression of candidate nucleotide sequence in a subject sample of leukocytes. In some cases, e.g., when multiple methods are employed to determine expression patterns for a plurality of candidate nucleotide sequences, the recovered data, e.g., the expression profile, for the nucleotide sequences is a combination of quantitative and qualitative data.

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In some applications, expression of the plurality of candidate nucleotide sequences is evaluated sequentially. This is typically the case for methods that can be characterized as low- to moderate-throughput. In contrast, as the throughput of the elected assay increases, expression for the plurality of candidate nucleotide sequences in a sample or multiple samples of leukocytes, is assayed simultaneously. Again, the methods (and throughput) are largely determined by the individual practitioner, although, typically, it is preferable to employ methods that permit rapid, e.g. automated or partially automated, preparation and detection, on a scale that is time-efficient and cost-effective.

It is understood that the preceding discussion, while directed at the assessment of expression of the members of candidate libraries, is also applies to the assessment of the expression of members of diagnostic nucleotide sets, as further discussed below.

Genotyping

In addition to, or in conjunction with the correlation of expression profiles and clinical data, it is often desirable to correlate expression patterns with the subject's genotype at one or more genetic loci. The selected loci can be, for example, chromosomal loci corresponding to one or more member of the candidate library, polymorphic alleles for marker loci, or alternative disease related loci (not contributing to the candidate library) known to be, or putatively associated with, a disease (or disease criterion). Indeed, it will be appreciated, that where a (polymorphic) allele at a locus is linked to a disease (or to a predisposition to a disease), the presence of the allele can itself be a disease criterion.

Numerous well known methods exist for evaluating the genotype of an individual, including southern analysis, restriction fragment length polymorphism (RFLP) analysis, polymerase chain reaction (PCR), amplification length polymorphism (AFLP) analysis, single stranded conformation polymorphism (SSCP) analysis, single nucleotide polymorphism (SNP) analysis (e.g., via PCR, Taqman or molecular beacons), among many other useful methods. Many such procedures are readily adaptable to high throughput and/or automated (or semi-automated) sample preparation and analysis methods. Most, can be performed on nucleic acid samples recovered via simple procedures from the same sample of leukocytes as yielded the

material for expression profiling. Exemplary techniques are described in, e.g., Sambrook, and Ausubel, *supra*.

Identification of the diagnostic nucleotide sets of the invention

Identification of diagnostic nucleotide sets and disease specific target nucleotide sequence proceeds by correlating the leukocyte expression profiles with data regarding the subject's health status to produce a data set designated a "molecular signature." Examples of data regarding a patient's health status, also termed "disease criteria(ion)", is described below and in the Section titled "selected diseases," below. Methods useful for correlation analysis are further described elsewhere in the specification.

Generally, relevant data regarding the subject's health status includes retrospective or prospective health data, e.g., in the form of the subject's medical history, as provided by the subject, physician or third party, such as, medical diagnoses, laboratory test results, diagnostic test results, clinical events, or medication lists, as further described below. Such data may include information regarding a patient's response to treatment and/or a particular medication and data regarding the presence of previously characterized "risk factors." For example, cigarette smoking and obesity are previously identified risk factors for heart disease. Further examples of health status information, including diseases and disease criteria, is described in the section titled Selected diseases, below.

Typically, the data describes prior events and evaluations (i.e., retrospective data). However, it is envisioned that data collected subsequent to the sampling (i.e., prospective data) can also be correlated with the expression profile. The tissue sampled, e.g., peripheral blood, bronchial lavage, etc., can be obtained at one or more multiple time points and subject data is considered retrospective or prospective with respect to the time of sample procurement.

Data collected at multiple time points, called "longitudinal data", is often useful, and thus, the invention encompasses the analysis of patient data collected from the same patient at different time points. Analysis of paired samples, such as samples from a patient at different time, allows identification of differences that are specifically related to the disease state since the genetic variability specific to the patient is controlled for by the comparison. Additionally, other variables that exist between patients may be controlled for in this way, for example, the presence or

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absence of inflammatory diseases (e.g., rheumatoid arthritis) the use of medications that may effect leukocyte gene expression, the presence or absence of co-morbid conditions, etc. Methods for analysis of paired samples are further described below. Moreover, the analysis of a pattern of expression profiles (generated by collecting multiple expression profiles) provides information relating to changes in expression level over time, and may permit the determination of a rate of change, a trajectory, or an expression curve. Two longitudinal samples may provide information on the change in expression of a gene over time, while three longitudinal samples may be necessary to determine the "trajectory" of expression of a gene. Such information may be relevant to the diagnosis of a disease. For example, the expression of a gene may vary from individual to individual, but a clinical event, for example, a heart attack, may cause the level of expression to double in each patient. In this example, clinically interesting information is gleaned from the change in expression level, as opposed to the absolute level of expression in each individual.

Generally, small sample sizes of 10-40 samples from 10-20 individuals are used to identify a diagnostic nucleotide set. Larger sample sizes are generally necessary to validate the diagnostic nucleotide set for use in large and varied patient populations, as further described below. For example, extension of gene expression correlations to varied ethnic groups, demographic groups, nations, peoples or races may require expression correlation experiments on the population of interest.

Expression Reference Standards

Expression profiles derived from a patient (i.e., subjects diagnosed with, or exhibiting symptoms of, or exhibiting a disease criterion, or under a doctor's care for a disease) sample are compared to a control or standard expression RNA to facilitate comparison of expression profiles (e.g. of a set of candidate nucleotide sequences) from a group of patients relative to each other (i.e., from one patient in the group to other patients in the group, or to patients in another group).

For example, in one approach to identifying diagnostic nucleotide sets, expression profiles derived from patient samples are compared to a expression reference "standard." Standard expression reference can be, for example, RNA derived from resting cultured leukocytes or commercially available reference RNA, such as Universal reference RNA from Stratagene. *See Nature*, V406, 8-17-00, p. 747-752. Use of an expression reference standard is particularly useful when the expression of large numbers of nucleotide sequences is assayed, e.g. in an array, and

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in certain other applications, e.g. qualitative PCR, RT-PCR, etc., where it is desirable to compare a sample profile to a standard profile, and/or when large numbers of expression profiles, e.g. a patient population, are to be compared. Generally, an expression reference standard should be available in large quantities, should be a good substrate for amplification and labeling reactions, and should be capable of detecting a large percentage of candidate nucleic acids using suitable expression profiling technology.

Alternatively, or in addition, the expression profile derived from a patient sample is compared with the expression of an internal reference control gene, for example, β -actin or CD4. The relative expression of the profiled genes and the internal reference control gene (from the same individual) is obtained. An internal reference control may also be used with a reference RNA. For example, an expression profile for "gene 1" and the gene encoding CD4 can be determined in a patient sample and in a reference RNA. The expression of each gene can be expressed as the "relative" ratio of expression the gene in the patient sample compared with expression of the gene in the reference RNA. The expression ratio (sample/reference) for gene 1 may be divided by the expression ratio for CD4 (sample/reference) and thus the relative expression of gene 1 to CD4 is obtained.

The invention also provides a buffy coat control RNA useful for expression profiling, and a method of using control RNA produced from a population of buffy coat cells, the white blood cell layer derived from the centrifugation of whole blood. Buffy coat contains all white blood cells, including granulocytes, mononuclear cells and platelets. The invention also provides a method of preparing control RNA from buffy coat cells for use in expression profile analysis of leukocytes. Buffy coat fractions are obtained, e.g. from a blood bank or directly from individuals, preferably from a large number of individuals such that bias from individual samples is avoided and so that the RNA sample represents an average expression of a healthy population. Buffy coat fractions from about 50 or about 100, or more individuals are preferred. 10 ml buffy coat from each individual is used. Buffy coat samples are treated with an erythrocyte lysis buffer, so that erythrocytes are selectively removed. The leukocytes of the buffy coat layer are collected by centrifugation. Alternatively, the buffy cell sample can be further enriched for a particular leukocyte sub-populations, e.g. mononuclear cells, T-lymphocytes, etc. To enrich for mononuclear cells, the

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buffy cell pellet, above, is diluted in PBS (phosphate buffered saline) and loaded onto a non-polystyrene tube containing a polysucrose and sodium diatrizoate solution adjusted to a density of 1.077+/-0.001 g/ml. To enrich for T-lymphocytes, 45 ml of whole blood is treated with RosetteSep (Stem Cell Technologies), and incubated at room temperature for 20 minutes. The mixture is diluted with an equal volume of PBS plus 2% FBS and mixed by inversion. 30 ml of diluted mixture is layered on top of 15 ml DML medium (Stem Cell Technologies). The tube is centrifuged at 1200 x g, and the enriched cell layer at the plasma : medium interface is removed, washed with PBS + 2% FBS, and cells collected by centrifugation at 1200 x g. The cell pellet is treated with 5 ml of erythrocyte lysis buffer (EL buffer, Qiagen) for 10 minutes on ice, and enriched T-lymphocytes are collected by centrifugation.

In addition or alternatively, the buffy cells (whole buffy coat or sub-population, e.g. mononuclear fraction) can be cultured *in vitro* and subjected to stimulation with cytokines or activating chemicals such as phorbol esters or ionomycin. Such stimuli may increase expression of nucleotide sequences that are expressed in activated immune cells and might be of interest for leukocyte expression profiling experiments.

Following sub-population selection and/or further treatment, e.g. stimulation as described above, RNA is prepared using standard methods. For example, cells are pelleted and lysed with a phenol/guanidinium thiocyanate and RNA is prepared. RNA can also be isolated using a silica gel-based purification column or the column method can be used on RNA isolated by the phenol/guanidinium thiocyanate method. RNA from individual buffy coat samples can be pooled during this process, so that the resulting reference RNA represents the RNA of many individuals and individual bias is minimized or eliminated. In addition, a new batch of buffy coat reference RNA can be directly compared to the last batch to ensure similar expression pattern from one batch to another, using methods of collecting and comparing expression profiles described above/below. One or more expression reference controls are used in an experiment. For example, RNA derived from one or more of the following sources can be used as controls for an experiment: stimulated or unstimulated whole buffy coat, stimulated or unstimulated peripheral mononuclear cells, or stimulated or unstimulated T-lymphocytes.

Alternatively, the expression reference standard can be derived from any subject or class of subjects including healthy subjects or subjects diagnosed with the

same or a different disease or disease criterion. Expression profiles from subjects in two distinct classes are compared to determine which subset of nucleotide sequences in the candidate library best distinguish between the two subject classes, as further discussed below. It will be appreciated that in the present context, the term "distinct classes" is relevant to at least one distinguishable criterion relevant to a disease of interest, a "disease criterion." The classes can, of course, demonstrate significant overlap (or identity) with respect to other disease criteria, or with respect to disease diagnoses, prognoses, or the like. The mode of discovery involves, e.g., comparing the molecular signature of different subject classes to each other (such as patient to control, patients with a first diagnosis to patients with a second diagnosis, etc.) or by comparing the molecular signatures of a single individual taken at different time points. The invention can be applied to a broad range of diseases, disease criteria, conditions and other clinical and/or epidemiological questions, as further discussed above/below.

It is appreciated that while the present discussion pertains to the use of expression reference controls while identifying diagnostic nucleotide sets, expression reference controls are also useful during use of diagnostic nucleotide sets, e.g. use of a diagnostic nucleotide set for diagnosis of a disease, as further described below.

Analysis of expression profiles

In order to facilitate ready access, e.g., for comparison, review, recovery, and/or modification, the molecular signatures/expression profiles are typically recorded in a database. Most typically, the database is a relational database accessible by a computational device, although other formats, e.g., manually accessible indexed files of expression profiles as photographs, analogue or digital imaging readouts, spreadsheets, etc. can be used. Further details regarding preferred embodiments are provided below. Regardless of whether the expression patterns initially recorded are analog or digital in nature and/or whether they represent quantitative or qualitative differences in expression, the expression patterns, expression profiles (collective expression patterns), and molecular signatures (correlated expression patterns) are stored digitally and accessed via a database. Typically, the database is compiled and maintained at a central facility, with access being available locally and/or remotely.

As additional samples are obtained, and their expression profiles determined and correlated with relevant subject data, the ensuing molecular signatures are likewise recorded in the database. However, rather than each subsequent addition

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being added in an essentially passive manner in which the data from one sample has little relation to data from a second (prior or subsequent) sample, the algorithms optionally additionally query additional samples against the existing database to further refine the association between a molecular signature and disease criterion. Furthermore, the data set comprising the one (or more) molecular signatures is optionally queried against an expanding set of additional or other disease criteria. The use of the database in integrated systems and web embodiments is further described below.

Analysis of expression profile data from arrays

Expression data is analyzed using methods well known in the art, including the software packages Imagene (Biodiscovery, Marina del Rey, CA), Feature Extraction (Agilent, Palo Alto, CA), and Scanalyze (Stanford University). In the discussion that follows, a "feature" refers to an individual spot of DNA on an array. Each gene may have more than one feature. For example, hybridized microarrays are scanned and analyzed on an Axon Instruments scanner using GenePix 3.0 software (Axon Instruments, Union City, CA). The data extracted by GenePix is used for all downstream quality control and expression evaluation. The data is derived as follows. The data for all features flagged as "not found" by the software is removed from the dataset for individual hybridizations. The "not found" flag by GenePix indicates that the software was unable to discriminate the feature from the background. Each feature is examined to determine the value of its signal. The median pixel intensity of the background (B_n) is subtracted from the median pixel intensity of the feature (F_n) to produce the background-subtracted signal (hereinafter, "BGSS"). The BGSS is divided by the standard deviation of the background pixels to provide the signal-to-noise ratio (hereinafter, "S/N"). Features with a S/N of three or greater in both the Cy3 channel (corresponding to the sample RNA) and Cy5 channel (corresponding to the reference RNA) are used for further analysis (hereinafter denoted "useable features"). Alternatively, different S/Ns are used for selecting expression data for an analysis. For example, only expression data with signal to noise ratios > 3 might be used in an analysis.

For each usable feature (i), the expression level (e) is expressed as the logarithm of the ratio (R) of the Background Subtracted Signal (hereinafter "BGSS") for the Cy3 (sample RNA) channel divided by the BGSS for the Cy5 channel (reference RNA). This "log ratio" value is used for comparison to other experiments.

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$$R_i = \frac{BGSS_{sample}}{BGSS_{reference}} \quad (0.1)$$

$$e_i = \log r_i \quad (0.2)$$

Variation in signal across hybridizations may be caused by a number of factors affecting hybridization, DNA spotting, wash conditions, and labeling efficiency.

A single reference RNA may be used with all of the experimental RNAs, permitting multiple comparisons in addition to individual comparisons. By comparing sample RNAs to the same reference, the gene expression levels from each sample are compared across arrays, permitting the use of a consistent denominator for our experimental ratios.

Scaling

The data may be scaled (normalized) to control for labeling and hybridization variability within the experiment, using methods known in the art. Scaling is desirable because it facilitates the comparison of data between different experiments, patients, etc. Generally the BGSS are scaled to a factor such as the median, the mean, the trimmed mean, and percentile. Additional methods of scaling include: to scale between 0 and 1, to subtract the mean, or to subtract the median.

Scaling is also performed by comparison to expression patterns obtained using a common reference RNA, as described in greater detail above. As with other scaling methods, the reference RNA facilitates multiple comparisons of the expression data, e.g., between patients, between samples, etc. Use of a reference RNA provides a consistent denominator for experimental ratios.

In addition to the use of a reference RNA, individual expression levels may be adjusted to correct for differences in labeling efficiency between different hybridization experiments, allowing direct comparison between experiments with different overall signal intensities, for example. A scaling factor (a) may be used to adjust individual expression levels as follows. The median of the scaling factor (a), for example, BGSS, is determined for the set of all features with a S/N greater than three. Next, the BGSS_{*i*} (the BGSS for each feature "i") is divided by the median for

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all features (a), generating a scaled ratio. The scaled ratio is used to determine the expression value for the feature (e_i), or the log ratio.

$$S_i = \frac{BGSS_i}{a} \quad (0.3)$$

$$e_i = \log \left(\frac{Cy3S_i}{Cy5S_i} \right) \quad (0.4)$$

In addition, or alternatively, control features are used to normalize the data for labeling and hybridization variability within the experiment. Control feature may be cDNA for genes from the plant, *Arabidopsis thaliana*, that are included when spotting the mini-array. Equal amounts of RNA complementary to control cDNAs are added to each of the samples before they were labeled. Using the signal from these control genes, a normalization constant (L) is determined according to the following formula:

$$L_j = \frac{\frac{\sum_{i=1}^N BGSS_{j,i}}{N}}{\frac{\sum_{j=1}^K \frac{\sum_{i=1}^N BGSS_{j,i}}{N}}{K}}$$

where $BGSS_i$ is the signal for a specific feature, N is the number of *A. thaliana* control features, K is the number of hybridizations, and L_j is the normalization constant for each individual hybridization.

Using the formula above, the mean for all control features of a particular hybridization and dye (e.g., Cy3) is calculated. The control feature means for all Cy3 hybridizations are averaged, and the control feature mean in one hybridization divided by the average of all hybridizations to generate a normalization constant for that particular Cy3 hybridization (L_j), which is used as a in equation (0.3). The same normalization steps may be performed for Cy3 and Cy5 values.

Many additional methods for normalization exist and can be applied to the data. In one method, the average ratio of Cy3 BGSS / Cy5 BGSS is determined for all features on an array. This ratio is then scaled to some arbitrary number, such as 1 or some other number. The ratio for each probe is then multiplied by the scaling

factor required to bring the average ratio to the chosen level. This is performed for each array in an analysis. Alternatively, the ratios are normalized to the average ratio across all arrays in an analysis.

Correlation analysis

Correlation analysis is performed to determine which array probes have expression behavior that best distinguishes or serves as markers for relevant groups of samples representing a particular clinical condition. Correlation analysis, or comparison among samples representing different disease criteria (e.g., clinical conditions), is performed using standard statistical methods. Numerous algorithms are useful for correlation analysis of expression data, and the selection of algorithms depends in part on the data analysis to be performed. For example, algorithms can be used to identify the single most informative gene with expression behavior that reliably classifies samples, or to identify all the genes useful to classify samples. Alternatively, algorithms can be applied that determine which set of 2 or more genes have collective expression behavior that accurately classifies samples. The use of multiple expression markers for diagnostics may overcome the variability in expression of a gene between individuals, or overcome the variability intrinsic to the assay. Multiple expression markers may include redundant markers, in that two or more genes or probes may provide the same information with respect to diagnosis. This may occur, for example, when two or more genes or gene probes are coordinately expressed. It will be appreciated that while the discussion above pertains to the analysis of RNA expression profiles the discussion is equally applicable to the analysis of profiles of proteins or other molecular markers.

Prior to analysis, expression profile data may be formatted or prepared for analysis using methods known in the art. For example, often the log ratio of scaled expression data for every array probe is calculated using the following formula:

$\log(\text{Cy 3 BGSS} / \text{Cy5 BGSS})$, where Cy 3 signal corresponds to the expression of the gene in the clinical sample, and Cy5 signal corresponds to expression of the gene in the reference RNA.

Data may be further filtered depending on the specific analysis to be done as noted below. For example, filtering may be aimed at selecting only samples with expression above a certain level, or probes with variability above a certain level between sample sets.

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The following non-limiting discussion consider several statistical methods known in the art. Briefly, the t-test and ANOVA are used to identify single genes with expression differences between or among populations, respectively. Multivariate methods are used to identify a set of two or more genes for which expression discriminates between two disease states more specifically than expression of any single gene.

t-test

The simplest measure of a difference between two groups is the Student's t test. See, e.g., Welsh et al. (2001) Proc Natl Acad Sci USA 98:1176-81 (demonstrating the use of an unpaired Student's t-test for the discovery of differential gene expression in ovarian cancer samples and control tissue samples). The t- test assumes equal variance and normally distributed data. This test identifies the probability that there is a difference in expression of a single gene between two groups of samples. The number of samples within each group that is required to achieve statistical significance is dependent upon the variation among the samples within each group. The standard formula for a t-test is:

$$t(e_i) = \frac{\bar{e}_{i,c} - \bar{e}_{i,t}}{\sqrt{(s_{i,c}^2/n_c) + (s_{i,t}^2/n_t)}}, \quad (0.5)$$

where \bar{e}_i is the difference between the mean expression level of gene i in groups c and t, $s_{i,c}$ is the variance of gene x in group c and $s_{i,t}$ is the variance of gene x in group t. n_c and n_t are the numbers of samples in groups c and t.

The combination of the t statistic and the degrees of freedom [$\min(n_t, n_c)-1$] provides a p value, the probability of rejecting the null hypothesis. A p-value of ≤ 0.01 , signifying a 99 percent probability the mean expression levels are different between the two groups (a 1% chance that the mean expression levels are in fact not different and that the observed difference occurred by statistical chance), is often considered acceptable.

When performing tests on a large scale, for example, on a large dataset of about 8000 genes, a correction factor must be included to adjust for the number of individual tests being performed. The most common and simplest correction is the

Bonferroni correction for multiple tests, which divides the p-value by the number of tests run. Using this test on an 8000 member dataset indicates that a p value of ≤ 0.00000125 is required to identify genes that are likely to be truly different between the two test conditions.

Wilcoxon's signed ranks test

This method is non-parametric and is utilized for paired comparisons. See e.g., Sokal and Rohlf (1987) Introduction to Biostatistics 2nd edition, WH Freeman, New York. At least 6 pairs are necessary to apply this statistic. This test is useful for analysis of paired expression data (for example, a set of patients who have cardiac transplant biopsy on 2 occasions and have a grade 0 on one occasion and a grade 3A on another).

ANOVA

Differences in gene expression across multiple related groups may be assessed using an Analysis of Variance (ANOVA), a method well known in the art (Michelson and Schofield, 1996).

Multivariate analysis

Many algorithms suitable for multivariate analysis are known in the art. Generally, a set of two or more genes for which expression discriminates between two disease states more specifically than expression of any single gene is identified by searching through the possible combinations of genes using a criterion for discrimination, for example the expression of gene X must increase from normal 300 percent, while the expression of genes Y and Z must decrease from normal by 75 percent. Ordinarily, the search starts with a single gene, then adds the next best fit at each step of the search. Alternatively, the search starts with all of the genes and genes that do not aid in the discrimination are eliminated step-wise.

Paired samples

Paired samples, or samples collected at different time-points from the same patient, are often useful, as described above. For example, use of paired samples permits the reduction of variation due to genetic variation among individuals. In addition, the use of paired samples has a statistical significance, in that data derived from paired samples can be calculated in a different manner that recognizes the reduced variability. For example, the formula for a t-test for paired samples is:

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$$t(e_x) = \frac{\bar{D}_{e_x}}{\sqrt{\frac{\sum D^2 - (\sum D)^2 / b}{b-1}}}, \quad (0.5)$$

where D is the difference between each set of paired samples and b is the number of sample pairs. \bar{D} is the mean of the differences between the members of the pairs. In this test, only the differences between the paired samples are considered, then grouped together (as opposed to taking all possible differences between groups, as would be the case with an ordinary t-test). Additional statistical tests useful with paired data, e.g., ANOVA and Wilcoxon's signed rank test, are discussed above.

Diagnostic classification

Once a discriminating set of genes is identified, the diagnostic classifier (a mathematical function that assigns samples to diagnostic categories based on expression data) is applied to unknown sample expression levels.

Methods that can be used for this analysis include the following non-limiting list:

CLEAVER is an algorithm used for classification of useful expression profile data. See Raychaudhuri et al. (2001) Trends Biotechnol 19:189-193. CLEAVER uses positive training samples (e.g., expression profiles from samples known to be derived from a particular patient or sample diagnostic category, disease or disease criteria), negative training samples (e.g., expression profiles from samples known not to be derived from a particular patient or sample diagnostic category, disease or disease criteria) and test samples (e.g., expression profiles obtained from a patient), and determines whether the test sample correlates with the particular disease or disease criteria, or does not correlate with a particular disease or disease criteria. CLEAVER also generates a list of the 20 most predictive genes for classification.

Artificial neural networks (hereinafter, "ANN") can be used to recognize patterns in complex data sets and can discover expression criteria that classify samples into more than 2 groups. The use of artificial neural networks for discovery of gene expression diagnostics for cancers using expression data generated by oligonucleotide expression microarrays is demonstrated by Khan et al. (2001) Nature Med. 7:673-9. Khan found that 96 genes provided 0% error rate in classification of the tumors. The most important of these genes for classification was then determined

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by measuring the sensitivity of the classification to a change in expression of each gene. Hierarchical clustering using the 96 genes results in correct grouping of the cancers into diagnostic categories.

Golub uses cDNA microarrays and a distinction calculation to identify genes with expression behavior that distinguishes myeloid and lymphoid leukemias. See Golub et al. (1999) Science 286:531-7. Self organizing maps were used for new class discovery. Cross validation was done with a "leave one out" analysis. 50 genes were identified as useful markers. This was reduced to as few as 10 genes with equivalent diagnostic accuracy.

Hierarchical and non-hierarchical clustering methods are also useful for identifying groups of genes that correlate with a subset of clinical samples such as with transplant rejection grade. Alizadeh used hierarchical clustering as the primary tool to distinguish different types of diffuse B-cell lymphomas based on gene expression profile data. See Alizadeh et al. (2000) Nature 403:503-11. Alizadeh used hierarchical clustering as the primary tool to distinguish different types of diffuse B-cell lymphomas based on gene expression profile data. A cDNA array carrying 17856 probes was used for these experiments, 96 samples were assessed on 128 arrays, and a set of 380 genes was identified as being useful for sample classification.

Perou demonstrates the use of hierarchical clustering for the molecular classification of breast tumor samples based on expression profile data. See Perou et al. (2000) Nature 406:747-52. In this work, a cDNA array carrying 8102 gene probes was used. 1753 of these genes were found to have high variation between breast tumors and were used for the analysis.

Hastie describes the use of gene shaving for discovery of expression markers. Hastie et al. (2000) Genome Biol. 1(2):RESEARCH 0003.1-0003.21. The gene shaving algorithm identifies sets of genes with similar or coherent expression patterns, but large variation across conditions (RNA samples, sample classes, patient classes). In this manner, genes with a tight expression pattern within a transplant rejection grade, but also with high variability across rejection grades are grouped together. The algorithm takes advantage of both characteristics in one grouping step. For example, gene shaving can identify useful marker genes with co-regulated expression. Sets of useful marker genes can be reduced to a smaller set, with each gene providing some non-redundant value in classification. This algorithm was used on the data set

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described in Alizadeh et al., supra, and the set of 380 informative gene markers was reduced to 234.

Selected Diseases

In principle, diagnostic nucleotide sets of the invention may be developed and applied to essentially any disease, or disease criterion, as long as at least one subset of nucleotide sequences is differentially expressed in samples derived from one or more individuals with a disease criteria or disease and one or more individuals without the disease criteria or disease, wherein the individual may be the same individual sampled at different points in time, or the individuals may be different individuals (or populations of individuals). For example, the subset of nucleotide sequences may be differentially expressed in the sampled tissues of subjects with the disease or disease criterion (e.g., a patient with a disease or disease criteria) as compared to subjects without the disease or disease criterion (e.g., patients without a disease (control patients)). Alternatively, or in addition, the subset of nucleotide sequence(s) may be differentially expressed in different samples taken from the same patient, e.g. at different points in time, at different disease stages, before and after a treatment, in the presence or absence of a risk factor, etc.

Expression profiles corresponding to sets of nucleotide sequences that correlate not with a diagnosis, but rather with a particular aspect of a disease can also be used to identify the diagnostic nucleotide sets and disease specific target nucleotide sequences of the invention. For example, such an aspect, or disease criterion, can relate to a subject's medical or family history, e.g., childhood illness, cause of death of a parent or other relative, prior surgery or other intervention, medications, symptoms (including onset and/or duration of symptoms), etc. Alternatively, the disease criterion can relate to a diagnosis, e.g., hypertension, diabetes, atherosclerosis, or prognosis (e.g., prediction of future diagnoses, events or complications), e.g., acute myocardial infarction, restenosis following angioplasty, reperfusion injury, allograft rejection, rheumatoid arthritis or systemic lupus erythematosus disease activity or the like. In other cases, the disease criterion corresponds to a therapeutic outcome, e.g., transplant rejection, bypass surgery or response to a medication, restenosis after stent implantation, collateral vessel growth due to therapeutic angiogenesis therapy, decreased angina due to revascularization, resolution of symptoms associated with a myriad of therapies, and the like. Alternatively, the disease criteria corresponds with

previously identified or classic risk factors and may correspond to prognosis or future disease diagnosis. As indicated above, a disease criterion can also correspond to genotype for one or more loci. Disease criteria (including patient data) may be collected (and compared) from the same patient at different points in time, from different patients, between patients with a disease (criterion) and patients representing a control population, etc. Longitudinal data, i.e., data collected at different time points from an individual (or group of individuals) may be used for comparisons of samples obtained from an individual (group of individuals) at different points in time, to permit identification of differences specifically related to the disease state, and to obtain information relating to the change in expression over time, including a rate of change or trajectory of expression over time. The usefulness of longitudinal data is further discussed in the section titled "Identification of diagnostic nucleotide sets of the invention".

It is further understood that diagnostic nucleotide sets may be developed for use in diagnosing conditions for which there is no present means of diagnosis. For example, in rheumatoid arthritis, joint destruction is often well under way before a patient experience symptoms of the condition. A diagnostic nucleotide set may be developed that diagnoses rheumatic joint destruction at an earlier stage than would be possible using present means of diagnosis, which rely in part on the presentation of symptoms by a patient. Diagnostic nucleotide sets may also be developed to replace or augment current diagnostic procedures. For example, the use of a diagnostic nucleotide set to diagnose cardiac allograft rejection may replace the current diagnostic test, a graft biopsy.

It is understood that the following discussion of diseases is exemplary and non-limiting, and further that the general criteria discussed above, e.g. use of family medical history, are generally applicable to the specific diseases discussed below.

In addition to leukocytes, as described throughout, the general method is applicable to nucleotide sequences that are differentially expressed in any subject tissue or cell type, by the collection and assessment of samples of that tissue or cell type. However, in many cases, collection of such samples presents significant technical or medical problems given the current state of the art.

Organ transplant rejection and success

A frequent complication of organ transplantation is recognition of the transplanted organ as foreign by the immune system resulting in rejection. Diagnostic

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nucleotide sets can be identified and validated for monitoring organ transplant success, rejection and treatment. Medications currently exist that suppress the immune system, and thereby decrease the rate of and severity of rejection. However, these drugs also suppress the physiologic immune responses, leaving the patient susceptible to a wide variety of opportunistic infections. At present there is no easy, reliable way to diagnose transplant rejection. Organ biopsy is the preferred method, but this is expensive, painful and associated with significant risk and has inadequate sensitivity for focal rejection.

Diagnostic nucleotide sets of the present invention can be developed and validated for use as diagnostic tests for transplant rejection and success. It is appreciated that the methods of identifying diagnostic nucleotide sets are applicable to any organ transplant population. For example, diagnostic nucleotide sets are developed for cardiac allograft rejection and success. In some cases, disease criteria correspond to acute stage rejection diagnosis based on organ biopsy and graded using the International Society for Heart and Lung Transplantation ("ISHLT") criteria. Other disease criteria correspond to information from the patient's medical history and information regarding the organ donor. Alternatively, disease criteria include the presence or absence of cytomegalovirus (CMV) infection, Epstein-Barr virus (EBV) infection, allograft dysfunction measured by physiological tests of cardiac function. (e.g., hemodynamic measurements from catheterization or echocardiograph data), and symptoms of other infections. Alternatively, disease criteria corresponds to therapeutic outcome, e.g. graft failure, re-transplantation, transplant vasculopathy, response to immunosuppressive medications, etc. Disease criteria may further correspond to a rejection episode of at least moderate histologic grade, which results in treatment of the patient with additional corticosteroids, anti-T cell antibodies, or total lymphoid irradiation; a rejection with histologic grade 2 or higher; a rejection with histologic grade ≤ 2 ; the absence of histologic rejection and normal or unchanged allograft function (based on hemodynamic measurements from catheterization or on echocardiographic data); the presence of severe allograft dysfunction or worsening allograft dysfunction during the study period (based on hemodynamic measurements from catheterization or on echocardiographic data); documented CMV infection by culture, histology, or PCR, and at least one clinical sign or symptom of infection; specific graft biopsy rejection grades; rejection of mild to moderate histologic severity prompting augmentation of the patient's chronic immunosuppressive regimen;

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rejection of mild to moderate severity with allograft dysfunction prompting plasmaphoresis or a diagnosis of "humoral" rejection; infections other than CMV, especially infection with Epstein Barr virus (EBV); lymphoproliferative disorder (also called post-transplant lymphoma); transplant vasculopathy diagnosed by increased intimal thickness on intravascular ultrasound (IVUS), angiography, or acute myocardial infarction; graft failure or retransplantation; and all cause mortality. Further specific examples of clinical data useful as disease criteria are provided in Example 11.

In another example, diagnostic nucleotide sets are developed and validated for use in treatment of kidney allograft rejection. Disease criteria correspond to, e.g., results of biopsy analysis for kidney allograft rejection, serum creatine level, and urinalysis results. Another disease criteria corresponds to the need for hemodialysis or other renal replacement therapy. Diagnostic nucleotide sets are developed and validated for use in diagnosis and treatment of bone marrow transplant rejection and liver transplant rejection, respectively. Disease criteria for bone marrow transplant rejection correspond to the diagnosis and monitoring of graft rejection and/or graft versus host disease. Disease criteria for liver transplant rejection include levels of serum markers for liver damage and liver function such as AST (aspartate aminotransferase), ALT (alanine aminotransferase), Alkaline phosphatase, GGT, (gamma-glutamyl transpeptidase) Bilirubin, Albumin and Prothrombin time. Further disease criteria correspond to hepatic encephalopathy, medication usage, ascites, and histological rejection on graft biopsy. In addition, urine can be utilized for at the target tissue for profiling in renal transplant, while biliary and intestinal and feces may be used favorably for hepatic or intestinal organ allograft rejection.

Atherosclerosis and Stable Angina Pectoris

Over 50 million patients in the U.S. have atherosclerotic coronary artery disease (hereinafter, "CAD"), and it is of great importance to identify patients who will suffer complications from the disease. Atherosclerosis leads to progressive narrowing of the coronary arteries, which may lead to myocardial ischemia, which manifests as stable angina pectoris, or chest pain with exertion. In addition to chest pain, patients may also have shortness of breath (dyspnea), fatigue, nausea or other symptoms with exertion. Myocardial infarction (heart attack) and unstable angina are acute events associated with atherosclerosis. There is currently no way to accurately predict the occurrence of acute events in patients with atherosclerosis, however.

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Although the presence of classic risk factors and arterial wall calcification (as assessed by CT scanning) is weakly correlated with the occurrence of acute coronary syndrome, the degree of artery stenosis (i.e. vessel occlusion as a result of atherosclerosis) correlates poorly with the occurrence of future acute events, as acute events occur more commonly in coronary arteries with 40-50% blockage than arteries that are 80-90% blocked. Coronary angiography can provide information about degree of coronary blockage, but is a poor tool for the measurement of disease activity and the prediction of the likelihood of acute events and other poor outcomes.

Diagnostic nucleotide sets are developed and validated for use in diagnosis and monitoring of atherosclerosis, and in predicting the likelihood of complications, e.g. angina and myocardial infarction. Alternatively, or in addition, disease criteria correspond to symptoms or diagnosis of disease progression, e.g. clinical results of angiography indicating progressive narrowing of vessel lumens. In another aspect, diagnostic nucleotide sets are developed for use in predicting the likelihood of future acute events in patients suffering from atherosclerosis. Disease criteria correspond to retrospective data, for example a recent history of unstable angina or myocardial infarction. Disease criteria also correspond to prospective data, for example, the occurrence of unstable angina or myocardial infarction. In another case, disease criteria correspond to standard medical indicators of occurrence of an acute event, e.g. serum enzyme levels, electrocardiographic testing, chest pain, nuclear magnetic imaging, etc.

Congestive Heart Failure

Congestive heart failure (hereinafter, "CHF") is a disease that affects increasing numbers of individuals. Without being bound by theory, it is believed that CHF is associated with systemic inflammation. Markers of systemic inflammation and serum cytokine levels such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) and serum cytokine levels are elevated (or altered) in patients with CHF, and elevation correlates with the severity and progression of the disease. Furthermore, serum catecholamine levels (epinephrine and norepinephrine) are also elevated in proportion to the severity of CHF, and may directly alter leukocyte expression patterns. Currently, echocardiography is the test primarily used to assess the severity of CHF and monitor progression of the disease. There are a number of drugs that are efficacious in treating CHF, such as beta-blockers and ACE inhibitors.

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A leukocyte test with the ability to determine the rate of progression and the adequacy of therapy is of great interest.

Diagnostic nucleotide sets are developed and validated for use in diagnosis and monitoring of progression and rate of progression (activity) of CHF. Disease criteria correspond to the results of echocardiography testing, which may indicate diagnosis of CHF or increasing severity of CHF as evidenced by worsening parameters for ventricular function, such as the ejection fraction, fractional shortening, wall motion or ventricular pressures. Alternatively, or in addition, disease criteria correspond to hospitalization for CHF, death, pulmonary edema, increased cardiac chamber dimensions on echocardiography or another imaging test, exercise testing of hemodynamic measurements, serial CRP, other serum markers, NYHA functional classes, quality of life measures, renal function, transplant listing, pulmonary edema, left ventricular assist device use, medication use and changes, and worsening of Ejection Fraction by echocardiography, angiography, MRI, CT or nuclear imaging.. In another aspect, disease criteria correspond to response to drug therapy, e.g. beta-blockers or ACE inhibitors.

Risk factors for coronary artery disease

The established and classic risks for the occurrence of coronary artery disease and complications of that disease are: cigarette smoking, diabetes, hypertension, hyperlipidemia and a family history of early atherosclerosis. Obesity, sedentary lifestyle, syndrome X, cocaine use, chronic hemodialysis and renal disease, radiation exposure, endothelial dysfunction, elevated plasma homocysteine, elevated plasma lipoprotein a, elevated CRP, infection with CMV and chlamydia infection are less well established, controversial, or putative risk factors for the disease. Risk factors are known to be associated with patient prognosis and outcome, but the contribution of each risk factor to the future clinical state of a patient is difficult to measure. The effect of risk factor modification (e.g., smoking cessation, treatment of hypercholesterolemia) on overall risk and future outcome is also difficult to quantify.

Diagnostic nucleotide sets may be developed that correlate with these risk factors, or the sum of the risk factors for use in predicting occurrence of coronary artery disease. Disease criteria correspond to risk factors, as exemplified above, as well as to occurrence of coronary artery disease. Alternatively, or in addition, disease criteria corresponding to risk factors may contribute to a numerical weighted average, which itself may be treated as a disease criteria and may be used for correlation to

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gene expression. In another aspect, risk factors may be modified in a patient, e.g. by behavioral change, or decrease cholesterol through chemotherapy in patients with hypocholesteremia. Disease criteria may further correspond to diagnosis of coronary disease.

Restenosis

Angioplasty can re-open a narrowed artery. However, the long-term success rate of these procedures is limited by restenosis, the re-narrowing of a coronary artery after an angioplasty. Currently, about 50% of treated arteries re-narrow after angioplasty and about 30% re-narrow after standard stent placement. Restenosis usually becomes apparent within 3 months of the angioplasty procedure. Presently, there is no reliable method for predicting which arteries will succumb to restenosis, though small vessels tend to be more likely to re-narrow, as do vessels of diabetics, renal patients and vessels exposed to high-pressure balloon inflation during balloon angioplasty.

Diagnostic nucleotide sets are developed and validated to predict restenosis in patients before undergoing angioplasty or shortly thereafter. Disease criteria correspond to angiogram testing (diagnosis of restenosis), as well as clinical symptoms of restenosis, e.g. chest pain due to re-narrowing of the artery, as confirmed by angiogram. Anti-restenotic drug therapy is also identified for each patient. The diagnostic nucleotide set are useful to identify patients about to undergo angioplasty who would benefit from stents, radiation-emitting stents, and anti-restenotic drug delivering stents. Patients that would benefit from post-angioplasty anti-restenotic drug therapy may also be identified.

Rheumatoid Arthritis

Rheumatoid arthritis (RA) affects about two million patients in the US and is a chronic and debilitating inflammatory arthritis, particularly involving pain and destruction of the joints. RA often goes undiagnosed because patients may have no pain, but the disease is actively destroying the joint. Other patients are known to have RA, and are treated to alleviate symptoms, but the rate of progression of joint destruction can't easily be monitored. Drug therapy is available, but the most effective medicines are toxic (e.g., steroids, methotrexate) and thus need to be used with caution. A new class of medications (TNF blockers) is very effective, but the drugs are expensive, have side effects, and not all patients respond. Side-effects are

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common and include immune suppression, toxicity to organ systems, allergy and metabolic disturbances.

Diagnostic nucleotide sets of the invention are developed and validated for use in diagnosis and treatment of RA. Disease criteria correspond to disease symptoms (e.g., joint pain, joint swelling and joint stiffness and any of the American College for Rheumatology criteria for the diagnosis of RA, see Arnett et al (1988) Arthr. Rheum. 31:315-24), progression of joint destruction (e.g. as measured by serial hand radiographs, assessment of joint function and mobility), surgery, need for medication, additional diagnoses of inflammatory and non-inflammatory conditions, and clinical laboratory measurements including complete blood counts with differentials, CRP, ESR, ANA, Serum IL6, Soluble CD40 ligand, LDL, HDL, Anti-DNA antibodies, rheumatoid factor, C3, C4, serum creatinine. In addition, or alternatively, disease criteria correspond to response to drug therapy and presence or absence of side-effects or measures of improvement exemplified by the American College of Rheumatology "20%" and "50%" response/improvement rates. See Felson et al (1995) Arthr Rheum 38:531-37. Diagnostic nucleotide sets are identified that monitor and predict disease progression including flaring (acute worsening of disease accompanied by joint pain or other symptoms), response to drug treatment and likelihood of side-effects.

In addition to peripheral leukocytes, surgical specimens of rheumatoid joints can be used for leukocyte expression profiling experiments. Members of diagnostic nucleotide sets are candidates for leukocyte target nucleotide sequences, e.g. as a candidate drug target for rheumatoid arthritis.

Systemic Lupus Erythematosus (SLE)

SLE is a chronic, systemic inflammatory disease characterized by dysregulation of the immune system, which effects up to 2 million patients in the US. Symptoms of SLE include rashes, joint pain, abnormal blood counts, renal dysfunction and damage, infections, CNS disorders, arthralgias and autoimmunity. Patients may also have early onset atherosclerosis.

Diagnostic nucleotide sets are identified and validated for use in diagnosis and monitoring of SLE activity and progression. Disease criteria correspond to clinical data, e.g. symptom rash, joint pain, malaise, rashes, blood counts (white and red), tests of renal function e.g. creatinine, blood urea nitrogen (hereinafter, "bun") creative clearance, data obtained from laboratory tests including complete blood counts with differentials, CRP, ESR, ANA, Serum IL6, Soluble CD40 ligand, LDL, HDL, Anti-

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DNA antibodies, rheumatoid factor, C3, C4, serum creatinine and any medication levels, the need for pain medications, cumulative doses or immunosuppressive therapy, symptoms or any manifestation of carotid atherosclerosis (e.g. ultrasound diagnosis or any other manifestations of the disease), data from surgical procedures such as gross operative findings and pathological evaluation of resected tissues and biopsies (e.g., renal, CNS), information on pharmacological therapy and treatment changes, clinical diagnoses of disease “flare”, hospitalizations, death, quantitative joint exams, results from health assessment questionnaires (HAQs), and other clinical measures of patient symptoms and disability. In addition, disease criteria correspond to the clinical score known as SLEDAI (Bombadier C, Gladman DD, Urowitz MB, Caron D, Chang CH and the Committee on Prognosis Studies in SLE: Derivation of the SLEDAI for Lupus Patients. Arthritis Rheum 35:630-640, 1992.). Diagnostic nucleotide sets may be useful for diagnosis of SLE, monitoring disease progression including progressive renal dysfunction, carotid atherosclerosis and CNS dysfunction, and predicting occurrence of side-effects, for example.

Dermatomyositis/Polymyositis

Dermatomyositis/Polymyositis is an autoimmune/inflammatory disease of muscle and skin. Disease criteria correspond to clinical markers of muscle damage (e.g. creatine kinase or myoglobin), muscle strength, symptoms, skin rash or muscle biopsy results.

Diabetes

Insulin dependent (type I) diabetes is caused by an autoimmune attack of insulin producing cells in the pancreas. The disease does not manifest until greater than 90% of the insulin producing cells are destroyed. Diagnostic nucleotide sets are developed and validated for use in detecting diabetes before it is clinically evident. Disease criteria correspond to future occurrence of diabetes, glucose tolerance, serum glucose level, and levels of hemoglobin A1c or other markers.

Inflammatory Bowel Disease (Crohn's and Ulcerative Colitis)

Inflammatory Bowel Disease, e.g., Crohn's Disease and Ulcerative Colitis, are chronic inflammatory diseases of the intestine. Together they effect at least 1 million in the US. Currently, diagnosis and monitoring is accomplished by intestinal endoscopy with or without a biopsy. Steroids and other immune suppressing drugs are useful in treating these diseases, but these drugs cause toxicity and severe side-effects. Diagnostic nucleotide sets are developed for use in diagnosis and monitoring

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of disease progression. Disease criteria correspond to clinical criteria, e.g. symptoms of abdominal or pelvic pain, diarrhea, fever and rectal bleeding. Alternatively, or in addition, disease criteria correspond to endoscopy results or bowel biopsy results.

Osteoarthritis

20-40 million patients in the US have osteoarthritis. Patient groups are heterogeneous, with a subset of patients having earlier onset, more aggressive joint damage, involving more inflammation (leukocyte infiltration) leukocyte diagnostics can be used to distinguish osteoarthritis from rheumatoid arthritis, define likelihood and degree of response to NSAID therapy (non-steroidal anti-inflammatory drugs). Rate of progression of joint damage can also be assessed. Diagnostic nucleotide sets may be developed for use in selection and titration of treatment therapies. Disease criteria correspond to response to therapy, and disease progression using certain therapies, need for joint surgery, joint pain and disability.

Asthma

Asthma is a chronic inflammatory disease of the lungs. Clinical symptoms include chronic or acute airflow obstruction. Patients are treated with inhaled steroids or bronchodilators or systemic steroids and other medication, and disease progression is monitored clinically using a peak air flow meter or formal pulmonary function tests. Even with these tests, it is difficult to predict which patients are at highest risk for acute worsening of airway obstruction (an "asthma attack"). Diagnostic nucleotide sets are developed for use in predicting likelihood of acute asthma attacks, and for use in choosing and titrating drug therapy. Disease criteria correspond to pulmonary function testing, peak flow meter measurements, ER visits, inhaler use, subjective patient assessment of response to therapy, hospitalization and need for steroids.

Other inflammatory diseases:

Other inflammatory disease suitable for development and use of diagnostic nucleotide sets are polymyalgia rheumatica, temporal arteritis, polyarteritis nodosa, Wegener's granulomatosis, Whipple's disease, heterotopic ossification, Periprosthetic Osteolysis, Sepsis/ARDS, scleroderma, Grave's disease, Hashimoto's thyroiditis, psoriasis numerous others (See Table 1).

Viral diseases

Diagnostic leukocyte nucleotide sets may be developed and validated for use in diagnosing viral disease. In another aspect, viral nucleotide sequences may be

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added to a leukocyte nucleotide set for use in diagnosis of viral diseases. Alternatively, viral nucleotide sets and leukocyte nucleotides sets may be used sequentially.

Epstein-Barr virus (EBV)

EBV causes a variety of diseases such as mononucleosis, B-cell lymphoma, and pharyngeal carcinoma. It infects mononuclear cells and circulating atypical lymphocytes are a common manifestation of infection. Peripheral leukocyte gene expression is altered by infection. Transplant recipients and patients who are immunosuppressed are at increased risk for EBV-associated lymphoma.

Diagnostic nucleotide sets may be developed and validated for use in diagnosis and monitoring of EBV. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. Alternatively, EBV nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing EBV. Disease criteria correspond with diagnosis of EBV, and, in patients who are EBV-sero-positive, presence (or prospective occurrence) of EBV-related illnesses such as mononucleosis, and EBV-associated lymphoma. Diagnostic nucleotide sets are useful for diagnosis of EBV, and prediction of occurrence of EBV-related illnesses.

Cytomegalovirus (CMV)

Cytomegalovirus cause inflammation and disease in almost any tissue, particularly the colon, lung, bone marrow and retina, and is a very important cause of disease in immunosuppressed patients, e.g. transplant, cancer, AIDS. Many patients are infected with or have been exposed to CMV, but not all patients develop clinical disease from the virus. Also, CMV negative recipients of allografts that come from CMV positive donors are at high risk for CMV infection. As immunosuppressive drugs are developed and used, it is increasingly important to identify patients with current or impending clinical CMV disease, because the potential benefit of immunosuppressive therapy must be balanced with the increased rate of clinical CMV infection and disease that may result from the use of immunosuppression therapy. CMV may also play a role in the occurrence of atherosclerosis or restenosis after angioplasty.

Diagnostic nucleotide sets are developed for use in diagnosis and monitoring of CMV infection or re-activation of CMV infection. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. In another aspect, CMV nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing CMV.

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Disease criteria correspond to diagnosis of CMV (e.g., sero-positive state) and presence of clinically active CMV. Disease criteria may also correspond to prospective data, e.g. the likelihood that CMV will become clinically active or impending clinical CMV infection. Antiviral medications are available and diagnostic nucleotide sets can be used to select patients for early treatment, chronic suppression or prophylaxis of CMV activity.

Hepatitis B and C

These chronic viral infections affect about 1.25 and 2.7 million patients in the US, respectively. Many patients are infected, but suffer no clinical manifestations. Some patients with infection go on to suffer from chronic liver failure, cirrhosis and hepatic carcinoma.

Diagnostic nucleotide sets are developed for use in diagnosis and monitoring of HBV or HCV infection. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. In another aspect, viral nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing the virus and monitoring progression of liver disease. Disease criteria correspond to diagnosis of the virus (e.g., sero-positive state or other disease symptoms). Alternatively, disease criteria correspond to liver damage, e.g., elevated alkaline phosphatase, ALT, AST or evidence of ongoing hepatic damage on liver biopsy. Alternatively, disease criteria correspond to serum liver tests (AST, ALT, Alkaline Phosphatase, GGT, PT, bilirubin), liver biopsy, liver ultrasound, viral load by serum PCR, cirrhosis, hepatic cancer, need for hospitalization or listing for liver transplant. Diagnostic nucleotide sets are used to diagnose HBV and HCV, and to predict likelihood of disease progression. Antiviral therapeutic usage, such as Interferon gamma and Ribavirin, can also be disease criteria.

HIV

HIV infects T cells and certainly causes alterations in leukocyte expression. Diagnostic nucleotide sets are developed for diagnosis and monitoring of HIV. In one aspect, the diagnostic nucleotide set is a leukocyte nucleotide set. In another aspect, viral nucleotide sequences are added to a leukocyte nucleotide set, for use in diagnosing the virus. Disease criteria correspond to diagnosis of the virus (e.g., sero-positive state). In addition, disease criteria correspond to viral load, CD4 T cell counts, opportunistic infection, response to antiretroviral therapy, progression to AIDS, rate of progression and the occurrence of other HIV related outcomes (e.g.,

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malignancy, CNS disturbance). Response to antiretrovirals may also be disease criteria.

Pharmacogenomics

Pharmacogenomics is the study of the individual propensity to respond to a particular drug therapy (combination of therapies). In this context, response can mean whether a particular drug will work on a particular patient, e.g. some patients respond to one drug but not to another drug. Response can also refer to the likelihood of successful treatment or the assessment of progress in treatment. Titration of drug therapy to a particular patient is also included in this description, e.g. different patients can respond to different doses of a given medication. This aspect may be important when drugs with side-effects or interactions with other drug therapies are contemplated.

Diagnostic nucleotide sets are developed and validated for use in assessing whether a patient will respond to a particular therapy and/or monitoring response of a patient to drug therapy(therapies). Disease criteria correspond to presence or absence of clinical symptoms or clinical endpoints, presence of side-effects or interaction with other drug(s). The diagnostic nucleotide set may further comprise nucleotide sequences that are targets of drug treatment or markers of active disease.

Validation and accuracy of diagnostic nucleotide set using correlation analysis

Prior to widespread application of the diagnostic probe sets of the invention, the predictive value of the probe set is validated.

Typically, the oligonucleotide sequence of each probe is confirmed, e.g. by DNA sequencing using an oligonucleotide-specific primer. Partial sequence obtained is generally sufficient to confirm the identity of the oligonucleotide probe. Alternatively, a complementary polynucleotide is fluorescently labeled and hybridized to the array, or to a different array containing a resynthesized version of the oligo nucleotide probe, and detection of the correct probe is confirmed.

Typically, validation is performed by statistically evaluating the accuracy of the correspondence between the molecular signature for a diagnostic probe set and a selected indicator. For example, the expression differential for a nucleotide sequence between two subject classes can be expressed as a simple ratio of relative expression. The expression of the nucleotide sequence in subjects with selected indicator can be

compared to the expression of that nucleotide sequence in subjects without the indicator, as described in the following equations.

$\sum E_x a_i / N = E_x A$ the average expression of nucleotide sequence x in the members of group A;

$\sum E_x b_i / M = E_x B$ the average expression of nucleotide sequence x in the members of group B;

$E_x A / E_x B = \Delta E_x AB$ the average differential expression of nucleotide sequence x between groups A

and B:

where \sum indicates a sum; E_x is the expression of nucleotide sequence x relative to a standard; a_i are the individual members of group A, group A has N members; b_i are the individual members of group B, group B has M members.

The expression of at least two nucleotide sequences, e.g., nucleotide sequence X and nucleotide sequence Y are measured relative to a standard in at least one subject of group A (e.g., with a disease) and group B (e.g., without the disease). Ideally, for purposes of validation the indicator is independent from (i.e., not assigned based upon) the expression pattern. Alternatively, a minimum threshold of gene expression for nucleotide sequences X and Y, relative to the standard, are designated for assignment to group A. For nucleotide sequence x, this threshold is designated ΔE_x , and for nucleotide sequence y, the threshold is designated ΔE_y .

The following formulas are used in the calculations below:

Sensitivity = (true positives / true positives + false negatives)

Specificity = (true negatives / true negatives + false positives)

If, for example, expression of nucleotide sequence x above a threshold: $x > \Delta E_x$, is observed for 80/100 subjects in group A and for 10/100 subjects in group B, the sensitivity of nucleotide sequence x for the assignment to group A, at the given expression threshold ΔE_x , is 80%, and the specificity is 90%.

If the expression of nucleotide sequence y is $> \Delta E_y$ in 80/100 subjects in group A, and in 10/100 subjects in group B, then, similarly the sensitivity of nucleotide sequence y for the assignment to group A at the given threshold ΔE_y is 80% and the specificity is 90%. If in addition, 60 of the 80 subjects in group A that meet the expression threshold for nucleotide sequence y also meet the expression threshold ΔE_x and that 5 of the 10 subjects in group B that meet the expression

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threshold for nucleotide sequence y also meet the expression threshold ΔEx , the sensitivity of the test ($x > \Delta Ex$ and $y > \Delta Ey$) for assignment of subjects to group A is 60% and the specificity is 95%.

Alternatively, if the criteria for assignment to group A are change to: Expression of $x > \Delta Ex$ or expression of $y > \Delta Ey$, the sensitivity approaches 100% and the specificity is 85%.

Clearly, the predictive accuracy of any diagnostic probe set is dependent on the minimum expression threshold selected. The expression of nucleotide sequence X (relative to a standard) is measured in subjects of groups A (with disease) and B (without disease). The minimum threshold of nucleotide sequence expression for x , required for assignment to group A is designated $\Delta Ex 1$.

If 90/100 patients in group A have expression of nucleotide sequence $x > \Delta Ex 1$ and 20/100 patients in group B have expression of nucleotide sequence $x > \Delta Ex 1$, then the sensitivity of the expression of nucleotide sequence x (using $\Delta Ex 1$ as a minimum expression threshold) for assignment of patients to group A will be 90% and the specificity will be 80%.

Altering the minimum expression threshold results in an alteration in the specificity and sensitivity of the nucleotide sequences in question. For example, if the minimum expression threshold of nucleotide sequence x for assignment of subjects to group A is lowered to $\Delta Ex 2$, such that 100/100 subjects in group A and 40/100 subjects in group B meet the threshold, then the sensitivity of the test for assignment of subjects to group A will be 100% and the specificity will be 60%.

Thus, for 2 nucleotide sequences X and Y : the expression of nucleotide sequence x and nucleotide sequence y (relative to a standard) are measured in subjects belonging to groups A (with disease) and B (without disease). Minimum thresholds of nucleotide sequence expression for nucleotide sequences X and Y (relative to common standards) are designated for assignment to group A. For nucleotide sequence x , this threshold is designated $\Delta Ex 1$ and for nucleotide sequence y , this threshold is designated $\Delta Ey 1$.

If in group A, 90/100 patients meet the minimum requirements of expression $\Delta Ex 1$ and $\Delta Ey 1$, and in group B, 10/100 subjects meet the minimum requirements of expression $\Delta Ex 1$ and $\Delta Ey 1$, then the sensitivity of the test for assignment of subjects to group A is 90% and the specificity is 90%.

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Increasing the minimum expression thresholds for X and Y to $\Delta Ex2$ and $\Delta Ey2$, such that in group A, 70/100 subjects meet the minimum requirements of expression $\Delta Ex2$ and $\Delta Ey2$, and in group B, 3/100 subjects meet the minimum requirements of expression $\Delta Ex2$ and $\Delta Ey2$. Now the sensitivity of the test for assignment of subjects to group A is 70% and the specificity is 97%.

If the criteria for assignment to group A is that the subject in question meets either threshold, $\Delta Ex2$ or $\Delta Ey2$, and it is found that 100/100 subjects in group A meet the criteria and 20/100 subjects in group B meet the criteria, then the sensitivity of the test for assignment to group A is 100% and the specificity is 80%.

Individual components of a diagnostic probe set each have a defined sensitivity and specificity for distinguishing between subject groups. Such individual nucleotide sequences can be employed in concert as a diagnostic probe set to increase the sensitivity and specificity of the evaluation. The database of molecular signatures is queried by algorithms to identify the set of nucleotide sequences (i.e., corresponding to members of the probe set) with the highest average differential expression between subject groups. Typically, as the number of nucleotide sequences in the diagnostic probe set increases, so does the predictive value, that is, the sensitivity and specificity of the probe set. When the probe sets are defined they may be used for diagnosis and patient monitoring as discussed below. The diagnostic sensitivity and specificity of the probe sets for the defined use can be determined for a given probe set with specified expression levels as demonstrated above. By altering the expression threshold required for the use of each nucleotide sequence as a diagnostic, the sensitivity and specificity of the probe set can be altered by the practitioner. For example, by lowering the magnitude of the expression differential threshold for each nucleotide sequence in the set, the sensitivity of the test will increase, but the specificity will decrease. As is apparent from the foregoing discussion, sensitivity and specificity are inversely related and the predictive accuracy of the probe set is continuous and dependent on the expression threshold set for each nucleotide sequence. Although sensitivity and specificity tend to have an inverse relationship when expression thresholds are altered, both parameters can be increased as nucleotide sequences with predictive value are added to the diagnostic nucleotide set. In addition a single or a few markers may not be reliable expression markers across a population of patients. This is because of the variability in expression and measurement of expression that exists between measurements, individuals and

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individuals over time. Inclusion of a large number of candidate nucleotide sequences or large numbers of nucleotide sequences in a diagnostic nucleotide set allows for this variability as not all nucleotide sequences need to meet a threshold for diagnosis.

Generally, more markers are better than a single marker. If many markers are used to make a diagnosis, the likelihood that all expression markers will not meet some thresholds based upon random variability is low and thus the test will give fewer false negatives.

It is appreciated that the desired diagnostic sensitivity and specificity of the diagnostic nucleotide set may vary depending on the intended use of the set. For example, in certain uses, high specificity and high sensitivity are desired. For example, a diagnostic nucleotide set for predicting which patient population may experience side effects may require high sensitivity so as to avoid treating such patients. In other settings, high sensitivity is desired, while reduced specificity may be tolerated. For example, in the case of a beneficial treatment with few side effects, it may be important to identify as many patients as possible (high sensitivity) who will respond to the drug, and treatment of some patients who will not respond is tolerated. In other settings, high specificity is desired and reduced sensitivity may be tolerated. For example, when identifying patients for an early-phase clinical trial, it is important to identify patients who may respond to the particular treatment. Lower sensitivity is tolerated in this setting as it merely results in reduced patients who enroll in the study or requires that more patients are screened for enrollment.

Methods of using diagnostic nucleotide sets.

The invention also provide methods of using the diagnostic nucleotide sets to: diagnose disease; assess severity of disease; predict future occurrence of disease; predict future complications of disease; determine disease prognosis; evaluate the patient's risk, or "stratify" a group of patients; assess response to current drug therapy; assess response to current non-pharmacological therapy; determine the most appropriate medication or treatment for the patient; predict whether a patient is likely to respond to a particular drug; and determine most appropriate additional diagnostic testing for the patient, among other clinically and epidemiologically relevant applications.

The nucleotide sets of the invention can be utilized for a variety of purposes by physicians, healthcare workers, hospitals, laboratories, patients, companies and

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other institutions. As indicated previously, essentially any disease, condition, or status for which at least one nucleotide sequence is differentially expressed in leukocyte populations (or sub-populations) can be evaluated, e.g., diagnosed, monitored, etc. using the diagnostic nucleotide sets and methods of the invention. In addition to assessing health status at an individual level, the diagnostic nucleotide sets of the present invention are suitable for evaluating subjects at a "population level," e.g., for epidemiological studies, or for population screening for a condition or disease.

Collection and preparation of sample

RNA, protein and/or DNA is prepared using methods well-known in the art, as further described herein. It is appreciated that subject samples collected for use in the methods of the invention are generally collected in a clinical setting, where delays may be introduced before RNA samples are prepared from the subject samples of whole blood, e.g. the blood sample may not be promptly delivered to the clinical lab for further processing. Further delay may be introduced in the clinical lab setting where multiple samples are generally being processed at any given time. For this reason, methods which feature lengthy incubations of intact leukocytes at room temperature are not preferred, because the expression profile of the leukocytes may change during this extended time period. For example, RNA can be isolated from whole blood using a phenol/guanidine isothiocyanate reagent or another direct whole-blood lysis method, as described in, e.g., U.S. Patent Nos. 5,346,994 and 4,843,155. This method may be less preferred under certain circumstances because the large majority of the RNA recovered from whole blood RNA extraction comes from erythrocytes since these cells outnumber leukocytes 1000:1. Care must be taken to ensure that the presence of erythrocyte RNA and protein does not introduce bias in the RNA expression profile data or lead to inadequate sensitivity or specificity of probes.

Alternatively, intact leukocytes may be collected from whole blood using a lysis buffer that selectively lyses erythrocytes, but not leukocytes, as described, e.g., in (U.S. Patent Nos. 5,973,137, and 6,020,186). Intact leukocytes are then collected by centrifugation, and leukocyte RNA is isolated using standard protocols, as described herein. However, this method does not allow isolation of sub-populations of leukocytes, e.g. mononuclear cells, which may be desired. In addition, the expression profile may change during the lengthy incubation in lysis buffer, especially

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in a busy clinical lab where large numbers of samples are being prepared at any given time.

Alternatively, specific leukocyte cell types can be separated using density gradient reagents (Boyum, A, 1968.). For example, mononuclear cells may be separated from whole blood using density gradient centrifugation, as described, e.g., in U.S. Patents Nos. 4190535, 4350593, 4751001, 4818418, and 5053134. Blood is drawn directly into a tube containing an anticoagulant and a density reagent (such as Ficoll or Percoll). Centrifugation of this tube results in separation of blood into an erythrocyte and granulocyte layer, a mononuclear cell suspension, and a plasma layer. The mononuclear cell layer is easily removed and the cells can be collected by centrifugation, lysed, and frozen. Frozen samples are stable until RNA can be isolated. Density centrifugation, however, must be conducted at room temperature, and if processing is unduly lengthy, such as in a busy clinical lab, the expression profile may change.

The quality and quantity of each clinical RNA sample is desirably checked before amplification and labeling for array hybridization, using methods known in the art. For example, one microliter of each sample may be analyzed on a Bioanalyzer (Agilent 2100 Palo Alto, CA. USA) using an RNA 6000 nano LabChip (Caliper, Mountain View, CA. USA). Degraded RNA is identified by the reduction of the 28S to 18S ribosomal RNA ratio and/or the presence of large quantities of RNA in the 25-100 nucleotide range.

It is appreciated that the RNA sample for use with a diagnostic nucleotide set may be produced from the same or a different cell population, sub-population and/or cell type as used to identify the diagnostic nucleotide set. For example, a diagnostic nucleotide set identified using RNA extracted from mononuclear cells may be suitable for analysis of RNA extracted from whole blood or mononuclear cells, depending on the particular characteristics of the members of the diagnostic nucleotide set. Generally, diagnostic nucleotide sets must be tested and validated when used with RNA derived from a different cell population, sub-population or cell type than that used when obtaining the diagnostic gene set. Factors such as the cell-specific gene expression of diagnostic nucleotide set members, redundancy of the information provided by members of the diagnostic nucleotide set, expression level of the member of the diagnostic nucleotide set, and cell-specific alteration of expression of a member of the diagnostic nucleotide set will contribute to the usefulness of using a different

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RNA source than that used when identifying the members of the diagnostic nucleotide set. It is appreciated that it may be desirable to assay RNA derived from whole blood, obviating the need to isolate particular cell types from the blood.

Rapid method of RNA extraction suitable for production in a clinical setting of high quality RNA for expression profiling

In a clinical setting, obtaining high quality RNA preparations suitable for expression profiling, from a desired population of leukocytes poses certain technical challenges, including: the lack of capacity for rapid, high-throughput sample processing in the clinical setting, and the possibility that delay in processing (in a busy lab or in the clinical setting) may adversely affect RNA quality, e.g. by a permitting the expression profile of certain nucleotide sequences to shift. Also, use of toxic and expensive reagents, such as phenol, may be disfavored in the clinical setting due to the added expense associated with shipping and handling such reagents.

A useful method for RNA isolation for leukocyte expression profiling would allow the isolation of monocyte and lymphocyte RNA in a timely manner, while preserving the expression profiles of the cells, and allowing inexpensive production of reproducible high-quality RNA samples. Accordingly, the invention provides a method of adding inhibitor(s) of RNA transcription and/or inhibitor(s) of protein synthesis, such that the expression profile is "frozen" and RNA degradation is reduced. A desired leukocyte population or sub-population is then isolated, and the sample may be frozen or lysed before further processing to extract the RNA. Blood is drawn from subject population and exposed to ActinomycinD (to a final concentration of 10 ug/ml) to inhibit transcription, and cycloheximide (to a final concentration of 10 ug/ml) to inhibit protein synthesis. The inhibitor(s) can be injected into the blood collection tube in liquid form as soon as the blood is drawn, or the tube can be manufactured to contain either lyophilized inhibitors or inhibitors that are in solution with the anticoagulant. At this point, the blood sample can be stored at room temperature until the desired leukocyte population or sub-population is isolated, as described elsewhere. RNA is isolated using standard methods, e.g., as described above, or a cell pellet or extract can be frozen until further processing of RNA is convenient.

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The invention also provides a method of using a low-temperature density gradient for separation of a desired leukocyte sample. In another embodiment, the invention provides the combination of use of a low-temperature density gradient and the use of transcriptional and/or protein synthesis inhibitor(s). A desired leukocyte population is separated using a density gradient solution for cell separation that maintains the required density and viscosity for cell separation at 0-4°C. Blood is drawn into a tube containing this solution and may be refrigerated before and during processing as the low temperatures slow cellular processes and minimize expression profile changes. Leukocytes are separated, and RNA is isolated using standard methods. Alternately, a cell pellet or extract is frozen until further processing of RNA is convenient. Care must be taken to avoid rewarming the sample during further processing steps.

Alternatively, the invention provides a method of using low-temperature density gradient separation, combined with the use of actinomycin A and cyclohexamide, as described above.

Assessing expression for diagnostics

Expression profiles for the set of diagnostic nucleotide sequences in a subject sample can be evaluated by any technique that determines the expression of each component nucleotide sequence. Methods suitable for expression analysis are known in the art, and numerous examples are discussed in the Sections titled "Methods of obtaining expression data" and "high throughput expression Assays", above.

In many cases, evaluation of expression profiles is most efficiently, and cost effectively, performed by analyzing RNA expression. Alternatively, the proteins encoded by each component of the diagnostic nucleotide set are detected for diagnostic purposes by any technique capable of determining protein expression, e.g., as described above. Expression profiles can be assessed in subject leukocyte sample using the same or different techniques as those used to identify and validate the diagnostic nucleotide set. For example, a diagnostic nucleotide set identified as a subset of sequences on a cDNA microarray can be utilized for diagnostic (or prognostic, or monitoring, etc.) purposes on the same array from which they were identified. Alternatively, the diagnostic nucleotide sets for a given disease or condition can be organized onto a dedicated sub-array for the indicated purpose. It is important to note that if diagnostic nucleotide sets are discovered using one

technology, e.g. RNA expression profiling, but applied as a diagnostic using another technology, e.g. protein expression profiling, the nucleotide sets must generally be validated for diagnostic purposes with the new technology. In addition, it is appreciated that diagnostic nucleotide sets that are developed for one use, e.g. to diagnose a particular disease, may later be found to be useful for a different application, e.g. to predict the likelihood that the particular disease will occur. Generally, the diagnostic nucleotide set will need to be validated for use in the second circumstance. As discussed herein, the sequence of diagnostic nucleotide set members may be amplified from RNA or cDNA using methods known in the art providing specific amplification of the nucleotide sequences.

Identification of novel nucleotide sequences that are differentially expressed in leukocytes

Novel nucleotide sequences that are differentially expressed in leukocytes are also part of the invention. Previously unidentified open reading frames may be identified in a library of differentially expressed candidate nucleotide sequences, as described above, and the DNA and predicted protein sequence may be identified and characterized as noted above. We identified unnamed (not previously described as corresponding to a gene, or an expressed gene) nucleotide sequences in the our candidate nucleotide library, depicted in Table 3A, 3B and the sequence listing. Accordingly, further embodiments of the invention are the isolated nucleic acids described in Tables 3A and 3B, and in the sequence listing. The novel differentially expressed nucleotide sequences of the invention are useful in the diagnostic nucleotide set of the invention described above, and are further useful as members of a diagnostic nucleotide set immobilized on an array. The novel partial nucleotide sequences may be further characterized using sequence tools and publically or privately accessible sequence databases, as is well known in the art. Novel differentially expressed nucleotide sequences may be identified as disease target nucleotide sequences, described below. Novel nucleotide sequences may also be used as imaging reagent, as further described below.

As used herein, "novel nucleotide sequence" refers to (a) a nucleotide sequence containing at least one of the DNA sequences disclosed herein (as shown in FIGS. Table 3A, 3B and the sequence listing); (b) any DNA sequence that encodes the amino acid sequence encoded by the DNA sequences disclosed herein; (c) any

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DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, contained within the coding region of the nucleotide sequence to which the DNA sequences disclosed herein (as shown in Table 3A, 3B and the sequence listing) belong, under highly stringent conditions, e.g., hybridization to filter-bound DNA in 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA at 65° C, and washing in 0.1XSSC/0.1% SDS at 68° C. (Ausubel F. M. et al., eds., 1989, Current Protocols in Molecular Biology, Vol. I, Green Publishing Associates, Inc., and John Wiley & sons, Inc., New York, at p. 2.10.3), (d) any DNA sequence that hybridizes to the complement of the coding sequences disclosed herein, (as shown in Table 3A, 3B and the sequence listing) contained within the coding region of the nucleotide sequence to which DNA sequences disclosed herein (as shown in TABLES 3A, 3B and the sequence listing) belong, under less stringent conditions, such as moderately stringent conditions, e.g., washing in 0.2XSSC/0.1% SDS at 42°C. (Ausubel et al., 1989, supra), yet which still encodes a functionally equivalent gene product; and/or (e) any DNA sequence that is at least 90% identical, at least 80% identical or at least 70% identical to the coding sequences disclosed herein (as shown in TABLES 3A, 3B and the sequence listing), wherein % identity is determined using standard algorithms known in the art.

The invention also includes nucleic acid molecules, preferably DNA molecules, that hybridize to, and are therefore the complements of, the DNA sequences (a) through (c), in the preceding paragraph. Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing in 6xSSC/0.05% sodium pyrophosphate at 37°C. (for 14-base oligos), 48°C. (for 17-base oligos), 55°C. (for 20-base oligos), and 60°C. (for 23-base oligos). These nucleic acid molecules may act as target nucleotide sequence antisense molecules, useful, for example, in target nucleotide sequence regulation and/or as antisense primers in amplification reactions of target nucleotide sequence nucleic acid sequences. Further, such sequences may be used as part of ribozyme and/or triple helix sequences, also useful for target nucleotide sequence regulation. Still further, such molecules may be used as components of diagnostic methods whereby the presence of a disease-causing allele, may be detected.

The invention also encompasses (a) DNA vectors that contain any of the foregoing coding sequences and/or their complements (i.e., antisense); (b) DNA expression vectors that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences; and (c) genetically engineered host cells that contain any of the foregoing coding sequences operatively associated with a regulatory element that directs the expression of the coding sequences in the host cell. As used herein, regulatory elements include but are not limited to inducible and non-inducible promoters, enhancers, operators and other elements known to those skilled in the art that drive and regulate expression. The invention includes fragments of any of the DNA sequences disclosed herein. Fragments of the DNA sequences may be at least 5, at least 10, at least 15, at least 19 nucleotides, at least 25 nucleotides, at least 50 nucleotides, at least 100 nucleotides, at least 200, at least 500, or larger.

In addition to the nucleotide sequences described above, homologues of such sequences, as may, for example be present in other species, may be identified and may be readily isolated, without undue experimentation, by molecular biological techniques well known in the art, as well as use of gene analysis tools described above, and e.g., in Example 4. Further, there may exist nucleotide sequences at other genetic loci within the genome that encode proteins which have extensive homology to one or more domains of such gene products. These nucleotide sequences may also be identified via similar techniques.

For example, the isolated differentially expressed nucleotide sequence may be labeled and used to screen a cDNA library constructed from mRNA obtained from the organism of interest. Hybridization conditions will be of a lower stringency when the cDNA library was derived from an organism different from the type of organism from which the labeled sequence was derived. Alternatively, the labeled fragment may be used to screen a genomic library derived from the organism of interest, again, using appropriately stringent conditions. Such low stringency conditions will be well known to those of skill in the art, and will vary predictably depending on the specific organisms from which the library and the labeled sequences are derived. For guidance regarding such conditions see, for example, Sambrook et al., 1989, *Molecular Cloning, A Laboratory Manual*, Cold Springs Harbor Press, N.Y.; and Ausubel et al., 1989, *Current Protocols in Molecular Biology*, Green Publishing Associates and Wiley Interscience, N.Y.

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Novel nucleotide products include those proteins encoded by the novel nucleotide sequences described, above. Specifically, novel gene products may include polypeptides encoded by the novel nucleotide sequences contained in the coding regions of the nucleotide sequences to which DNA sequences disclosed herein (in TABLES 3A, 3B and the sequence listing).

In addition, novel protein products of novel nucleotide sequences may include proteins that represent functionally equivalent gene products. Such an equivalent novel gene product may contain deletions, additions or substitutions of amino acid residues within the amino acid sequence encoded by the novel nucleotide sequences described, above, but which result in a silent change, thus producing a functionally equivalent novel nucleotide sequence product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved.

For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid. "Functionally equivalent", as utilized herein, refers to a protein capable of exhibiting a substantially similar in vivo activity as the endogenous novel gene products encoded by the novel nucleotide described, above.

The novel gene products (protein products of the novel nucleotide sequences) may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing the novel gene polypeptides and peptides of the invention by expressing nucleic acid encoding novel nucleotide sequences are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing novel nucleotide sequence protein coding sequences and appropriate transcriptional/translational control signals. These methods include, for example, in vitro recombinant DNA techniques, synthetic techniques and in vivo recombination/genetic recombination. See, for example, the techniques described in Sambrook et al., 1989, supra, and Ausubel et al., 1989, supra. Alternatively, RNA capable of encoding novel nucleotide sequence protein sequences may be chemically synthesized using, for example, synthesizers. See, for example,

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the techniques described in "Oligonucleotide Synthesis", 1984, Gait, M. J. ed., IRL Press, Oxford.

A variety of host-expression vector systems may be utilized to express the novel nucleotide sequence coding sequences of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, exhibit the novel protein encoded by the novel nucleotide sequence of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (e.g., *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing novel nucleotide sequence protein coding sequences; yeast (e.g. *Saccharomyces*, *Pichia*) transformed with recombinant yeast expression vectors containing the novel nucleotide sequence protein coding sequences; insect cell systems infected with recombinant virus expression vectors (e.g., baculovirus) containing the novel nucleotide sequence protein coding sequences; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (e.g., Ti plasmid) containing novel nucleotide sequence protein coding sequences; or mammalian cell systems (e.g. COS, CHO, BHK, 293, 3T3) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (e.g., metallothionein promoter) or from mammalian viruses (e.g., the adenovirus late promoter; the vaccinia virus 7.5 K promoter).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the novel nucleotide sequence protein being expressed. For example, when a large quantity of such a protein is to be produced, for the generation of antibodies or to screen peptide libraries, for example, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:1791), in which the novel nucleotide sequence protein coding sequence may be ligated individually into the vector in frame with the *lac Z* coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 264:5503-5509); and the likes of pGEX vectors may

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also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target nucleotide sequence protein can be released from the GST moiety. Other systems useful in the invention include use of the FLAG epitope or the 6-HIS systems.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign nucleotide sequences. The virus grows in *Spodoptera frugiperda* cells. The novel nucleotide sequence coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter). Successful insertion of novel nucleotide sequence coding sequence will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda* cells in which the inserted nucleotide sequence is expressed. (E.g., see Smith et al., 1983, J. Virol. 46: 584; Smith, U.S. Pat. No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the novel nucleotide sequence coding sequence of interest may be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric nucleotide sequence may then be inserted in the adenovirus genome by in vitro or in vivo recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing novel nucleotide sequence encoded protein in infected hosts. (E.g., See Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 81:3655-3659). Specific initiation signals may also be required for efficient translation of inserted novel nucleotide sequence coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire novel nucleotide sequence, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the novel nucleotide sequence coding sequence is inserted, exogenous translational control signals, including,

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perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner et al., 1987, *Methods in Enzymol.* 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the product of the nucleotide sequence in the specific fashion desired. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, etc.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express the novel nucleotide sequence encoded protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (e.g., promoter, enhancer, sequences, transcription terminators, polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express novel nucleotide sequence encoded protein. Such engineered cell lines may be particularly useful in screening and evaluation of compounds that affect the endogenous activity of the novel nucleotide sequence encoded protein.

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A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, et al., 1977, Cell 11:223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska & Szybalski, 1962, Proc. Natl. Acad. Sci. USA 48:2026), and adenine phosphoribosyltransferase (Lowy, et al., 1980, Cell 22:817) genes can be employed in tk-, hgp^{rt}- or ap^{rt}- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, et al., 1980, Natl. Acad. Sci. USA 77:3567; O'Hare, et al., 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, et al., 1981, J. Mol. Biol. 150:1); and hyg^{ro}, which confers resistance to hygromycin (Santerre, et al., 1984, Gene 30:147) genes.

An alternative fusion protein system allows for the ready purification of non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8972-8976). In this system, the nucleotide sequence of interest is subcloned into a vaccinia recombination plasmid such that the nucleotide sequence's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni.sup.2+-nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Where recombinant DNA technology is used to produce the protein encoded by the novel nucleotide sequence for such assay systems, it may be advantageous to engineer fusion proteins that can facilitate labeling, immobilization and/or detection.

Indirect labeling involves the use of a protein, such as a labeled antibody, which specifically binds to the protein encoded by the novel nucleotide sequence. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and fragments produced by an Fab expression library.

The invention also provides for antibodies to the protein encoded by the novel nucleotide sequences. Described herein are methods for the production of antibodies capable of specifically recognizing one or more novel nucleotide sequence epitopes. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab')₂ fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above.

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Such antibodies may be used, for example, in the detection of a novel nucleotide sequence in a biological sample, or, alternatively, as a method for the inhibition of abnormal gene activity, for example, the inhibition of a disease target nucleotide sequence, as further described below. Thus, such antibodies may be utilized as part of cardiovascular or other disease treatment method, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of novel nucleotide sequence encoded proteins, or for the presence of abnormal forms of the such proteins.

For the production of antibodies to a novel nucleotide sequence, various host animals may be immunized by injection with a novel protein encoded by the novel nucleotide sequence, or a portion thereof. Such host animals may include but are not limited to rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as novel gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with novel gene product supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique which provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983, *Immunology Today* 4:72; Cole et al., 1983, *Proc. Natl. Acad. Sci. USA* 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, *Monoclonal Antibodies And Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD

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and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce novel nucleotide sequence-single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the F(ab')₂ fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Disease specific target nucleotide sequences

The invention also provides disease specific target nucleotide sequences, and sets of disease specific target nucleotide sequences. The diagnostic nucleotide sets, subsets thereof, novel nucleotide sequences, and individual members of the diagnostic nucleotide sets identified as described above are also disease specific target nucleotide sequences. In particular, individual nucleotide sequences that are differentially regulated or have predictive value that is strongly correlated with a disease or disease criterion are especially favorable as disease specific target nucleotide sequences. Sets of genes that are co-regulated may also be identified as disease specific target

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nucleotide sets. Such nucleotide sequences and/or nucleotide sequence products are targets for modulation by a variety of agents and techniques. For example, disease specific target nucleotide sequences (or the products of such nucleotide sequences, or sets of disease specific target nucleotide sequences) can be inhibited or activated by, e.g., target specific monoclonal antibodies or small molecule inhibitors, or delivery of the nucleotide sequence or gene product of the nucleotide sequence to patients. Also, sets of genes can be inhibited or activated by a variety of agents and techniques. The specific usefulness of the target nucleotide sequence(s) depends on the subject groups from which they were discovered, and the disease or disease criterion with which they correlate.

Imaging

The invention also provides for imaging reagents. The differentially expressed leukocyte nucleotide sequences, diagnostic nucleotide sets, or portions thereof, and novel nucleotide sequences of the invention are nucleotide sequences expressed in cells with or without disease. Leukocytes expressing a nucleotide sequence(s) that is differentially expressed in a disease condition may localize within the body to sites that are of interest for imaging purposes. For example, a leukocyte expressing a nucleotide sequence(s) that are differentially expressed in an individual having atherosclerosis may localize or accumulate at the site of an atherosclerotic plaque. Such leukocytes, when labeled, may provide a detection reagent for use in imaging regions of the body where labeled leukocyte accumulate or localize, for example, at the atherosclerotic plaque in the case of atherosclerosis. For example, leukocytes are collected from a subject, labeled in vitro, and reintroduced into a subject. Alternatively, the labeled reagent is introduced into the subject individual, and leukocyte labeling occurs within the patient.

Imaging agents that detect the imaging targets of the invention are produced by well-known molecular and immunological methods (for exemplary protocols, *see*, e.g., Ausubel, Berger, and Sambrook, as well as Harlow and Lane, *supra*).

For example, a full-length nucleic acid sequence, or alternatively, a gene fragment encoding an immunogenic peptide or polypeptide fragments, is cloned into a convenient expression vector, for example, a vector including an in-frame epitope or substrate binding tag to facilitate subsequent purification. Protein is then expressed from the cloned cDNA sequence and used to generate antibodies, or other specific

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binding molecules, to one or more antigens of the imaging target protein.

Alternatively, a natural or synthetic polypeptide (or peptide) or small molecule that specifically binds (or is specifically bound to) the expressed imaging target can be identified through well established techniques (*see, e.g.,* Mendel et al. (2000) Anticancer Drug Des 15:29-41; Wilson (2000) Curr Med Chem 7:73-98; Hamby and Showalter (1999) Pharmacol Ther 82:169-93; and Shimazawa et al. (1998) Curr Opin Struct Biol 8:451-8). The binding molecule, e.g., antibody, small molecule ligand, etc., is labeled with a contrast agent or other detectable label, e.g., gadolinium, iodine, or a gamma-emitting source. For in-vivo imaging of a disease process that involved leukocytes, the labeled antibody is infused into a subject, e.g., a human patient or animal subject, and a sufficient period of time is passed to permit binding of the antibody to target cells. The subject is then imaged with appropriate technology such as MRI (when the label is gadolinium) or with a gamma counter (when the label is a gamma emitter).

Identification of nucleotide sequence involved in leukocyte adhesion

The invention also encompasses a method of identifying nucleotide sequences involved in leukocyte adhesion. The interaction between the endothelial cell and leukocyte is a fundamental mechanism of all inflammatory disorders, including the diseases listed in Table 1. For example, the first visible abnormality in atherosclerosis is the adhesion to the endothelium and diapedesis of mononuclear cells (e.g., T-cell and monocyte). Insults to the endothelium (for example, cytokines, tobacco, diabetes, hypertension and many more) lead to endothelial cell activation. The endothelium then expresses adhesion molecules, which have counter receptors on mononuclear cells. Once the leukocyte receptors have bound the endothelial adhesion molecules, they stick to the endothelium, roll a short distance, stop and transmigrate across the endothelium. A similar set of events occurs in both acute and chronic inflammation.

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Human endothelial cells, e.g. derived from human coronary arteries, human aorta, human pulmonary artery, human umbilical vein or microvascular endothelial cells, are cultured as a confluent monolayer, using standard methods. Some of the endothelial cells are then exposed to cytokines or another activating stimuli such as oxidized LDL, hyperglycemia, shear stress, or hypoxia (Moser et al. 1992). Some endothelial cells are not exposed to such stimuli and serve as controls. For example, the endothelial cell monolayer is incubated with culture medium containing 5 U/ml of human recombinant IL-1alpha or 10 ng/ml TNF (tumor necrosis factor), for a period of minutes to overnight. The culture medium composition is changed or the flask is sealed to induce hypoxia. In addition, tissue culture plate is rotated to induce sheer stress.

Human T-cells and/or monocytes are cultured in tissue culture flasks or plates, with LGM-3 media from Clonetics. Cells are incubated at 37 degree C, 5% CO₂ and 95% humidity. These leukocytes are exposed to the activated or control endothelial layer by adding a suspension of leukocytes on to the endothelial cell monolayer. The endothelial cell monolayer is cultured on a tissue culture treated plate/ flask or on a microporous membrane. After a variable duration of exposures, the endothelial cells and leukocytes are harvested separately by treating all cells with trypsin and then sorting the endothelial cells from the leukocytes by magnetic affinity reagents to an endothelial cell specific marker such as PECAM-1 (Stem Cell Technologies). RNA is extracted from the isolated cells by standard techniques. Leukocyte RNA is labeled as described above, and hybridized to leukocyte candidate nucleotide library. Epithelial cell RNA is also labeled and hybridized to the leukocyte candidate nucleotide library. Alternatively, the epithelial cell RNA is hybridized to a epithelial cell candidate nucleotide library, prepared according to the methods described for leukocyte candidate libraries, above.

Hybridization to candidate nucleotide libraries will reveal nucleotide sequences that are up-regulated or down-regulated in leukocyte and/or epithelial cells undergoing adhesion. The differentially regulated nucleotide sequences are further characterized, e.g. by isolating and sequencing the full-length sequence, analysis of the DNA and predicted protein sequence, and functional characterization of the protein product of the nucleotide sequence, as described above. Further characterization may result in the identification of leukocyte adhesion specific target nucleotide sequences, which may be candidate targets for regulation of the

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inflammatory process. Small molecule or antibody inhibitors can be developed to inhibit the target nucleotide sequence function. Such inhibitors are tested for their ability to inhibit leukocyte adhesion in the in vitro test described above.

Integrated systems

Integrated systems for the collection and analysis of expression profiles, and molecular signatures, as well as for the compilation, storage and access of the databases of the invention, typically include a digital computer with software including an instruction set for sequence searching and analysis, and, optionally, high-throughput liquid control software, image analysis software, data interpretation software, a robotic control armature for transferring solutions from a source to a destination (such as a detection device) operably linked to the digital computer, an input device (e.g., a computer keyboard) for entering subject data to the digital computer, or to control analysis operations or high throughput sample transfer by the robotic control armature. Optionally, the integrated system further comprises an image scanner for digitizing label signals from labeled assay components, e.g., labeled nucleic acid hybridized to a candidate library microarray. The image scanner can interface with image analysis software to provide a measurement of the presence or intensity of the hybridized label, i.e., indicative of an on/off expression pattern or an increase or decrease in expression.

Readily available computational hardware resources using standard operating systems are fully adequate, e.g., a PC (Intel x86 or Pentium chip- compatible DOS,TM OS2,TM WINDOWS,TM WINDOWS NT,TM WINDOWS95,TM WINDOWS98,TM LINUX, or even Macintosh, Sun or PCs will suffice) for use in the integrated systems of the invention. Current art in software technology is similarly adequate (i.e., there are a multitude of mature programming languages and source code suppliers) for design, e.g., of an upgradeable open-architecture object-oriented heuristic algorithm, or instruction set for expression analysis, as described herein. For example, software for aligning or otherwise manipulating molecular signatures can be constructed by one of skill using a standard programming language such as Visual basic, Fortran, Basic, Java, or the like, according to the methods herein.

Various methods and algorithms, including genetic algorithms and neural networks, can be used to perform the data collection, correlation, and storage functions, as well as other desirable functions, as described herein. In addition, digital

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or analog systems such as digital or analog computer systems can control a variety of other functions such as the display and/or control of input and output files.

For example, standard desktop applications such as word processing software (e.g., Corel WordPerfect™ or Microsoft Word™) and database software (e.g., spreadsheet software such as Corel Quattro Pro™, Microsoft Excel™, or database programs such as Microsoft Access™ or Paradox™) can be adapted to the present invention by inputting one or more character string corresponding, e.g., to an expression pattern or profile, subject medical or historical data, molecular signature, or the like, into the software which is loaded into the memory of a digital system, and carrying out the operations indicated in an instruction set, e.g., as exemplified in Figure 2. For example, systems can include the foregoing software having the appropriate character string information, e.g., used in conjunction with a user interface in conjunction with a standard operating system such as a Windows, Macintosh or LINUX system. For example, an instruction set for manipulating strings of characters, either by programming the required operations into the applications or with the required operations performed manually by a user (or both). For example, specialized sequence alignment programs such as PILEUP or BLAST can also be incorporated into the systems of the invention, e.g., for alignment of nucleic acids or proteins (or corresponding character strings).

Software for performing the statistical methods required for the invention, e.g., to determine correlations between expression profiles and subsets of members of the diagnostic nucleotide libraries, such as programmed embodiments of the statistical methods described above, are also included in the computer systems of the invention. Alternatively, programming elements for performing such methods as principle component analysis (PCA) or least squares analysis can also be included in the digital system to identify relationships between data. Exemplary software for such methods is provided by Partek, Inc., St. Peter, Mo; <http://www.partek.com>.

Any controller or computer optionally includes a monitor which can include, e.g., a flat panel display (e.g., active matrix liquid crystal display, liquid crystal display), a cathode ray tube ("CRT") display, or another display system which serves as a user interface, e.g., to output predictive data. Computer circuitry, including numerous integrated circuit chips, such as a microprocessor, memory, interface circuits, and the like, is often placed in a casing or box which optionally also includes

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a hard disk drive, a floppy disk drive, a high capacity removable drive such as a writeable CD-ROM, and other common peripheral elements.

Inputting devices such as a keyboard, mouse, or touch sensitive screen, optionally provide for input from a user and for user selection, e.g., of sequences or data sets to be compared or otherwise manipulated in the relevant computer system. The computer typically includes appropriate software for receiving user instructions, either in the form of user input into a set parameter or data fields (e.g., to input relevant subject data), or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the system to carry out any desired operation.

The integrated system may also be embodied within the circuitry of an application specific integrated circuit (ASIC) or programmable logic device (PLD). In such a case, the invention is embodied in a computer readable descriptor language that can be used to create an ASIC or PLD. The integrated system can also be embodied within the circuitry or logic processors of a variety of other digital apparatus, such as PDAs, laptop computer systems, displays, image editing equipment, etc.

The digital system can comprise a learning component where expression profiles, and relevant subject data are compiled and monitored in conjunction with physical assays, and where correlations, e.g., molecular signatures with predictive value for a disease, are established or refined. Successful and unsuccessful combinations are optionally documented in a database to provide justification/preferences for user-base or digital system based selection of diagnostic nucleotide sets with high predictive accuracy for a specified disease or condition.

The integrated systems can also include an automated workstation. For example, such a workstation can prepare and analyze leukocyte RNA samples by performing a sequence of events including: preparing RNA from a human blood sample; labeling the RNA with an isotopic or non-isotopic label; hybridizing the labeled RNA to at least one array comprising all or part of the candidate library; and detecting the hybridization pattern. The hybridization pattern is digitized and recorded in the appropriate database.

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Automated RNA preparation tool

The invention also includes an automated RNA preparation tool for the preparation of mononuclear cells from whole blood samples, and preparation of RNA from the mononuclear cells. In a preferred embodiment, the use of the RNA preparation tool is fully automated, so that the cell separation and RNA isolation would require no human manipulations. Full automation is advantageous because it minimizes delay, and standardizes sample preparation across different laboratories. This standardization increases the reproducibility of the results.

Figure 2 depicts the processes performed by the RNA preparation tool of the invention. A primary component of the device is a centrifuge (A). Tubes of whole blood containing a density gradient solution, transcription/translation inhibitors, and a gel barrier that separates erythrocytes from mononuclear cells and serum after centrifugation are placed in the centrifuge (B). The barrier is permeable to erythrocytes and granulocytes during centrifugation, but does not allow mononuclear cells to pass through (or the barrier substance has a density such that mononuclear cells remain above the level of the barrier during the centrifugation). After centrifugation, the erythrocytes and granulocytes are trapped beneath the barrier, facilitating isolation of the mononuclear cell and serum layers. A mechanical arm removes the tube and inverts it to mix the mononuclear cell layer and the serum (C). The arm next pours the supernatant into a fresh tube (D), while the erythrocytes and granulocytes remained below the barrier. Alternatively, a needle is used to aspirate the supernatant and transfer it to a fresh tube. The mechanical arms of the device opens and closes lids, dispenses PBS to aid in the collection of the mononuclear cells by centrifugation, and moves the tubes in and out of the centrifuge. Following centrifugation, the supernatant is poured off or removed by a vacuum device (E), leaving an isolated mononuclear cell pellet. Purification of the RNA from the cells is performed automatically, with lysis buffer and other purification solutions (F) automatically dispensed and removed before and after centrifugation steps. The result is a purified RNA solution. In another embodiment, RNA isolation is performed using a column or filter method. In yet another embodiment, the invention includes an on-board homogenizer for use in cell lysis.

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Other automated systems

Automated and/or semi-automated methods for solid and liquid phase high-throughput sample preparation and evaluation are available, and supported by commercially available devices. For example, robotic devices for preparation of nucleic acids from bacterial colonies, e.g., to facilitate production and characterization of the candidate library include, for example, an automated colony picker (e.g., the Q-bot, Genetix, U.K.) capable of identifying, sampling, and inoculating up to 10,000/4 hrs different clones into 96 well microtiter dishes. Alternatively, or in addition, robotic systems for liquid handling are available from a variety of sources, e.g., automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, Beckman Coulter, Inc. (Fullerton, CA)) which mimic the manual operations performed by a scientist. Any of the above devices are suitable for use with the present invention, e.g., for high-throughput analysis of library components or subject leukocyte samples. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art.

High throughput screening systems that automate entire procedures, e.g., sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the relevant assay are commercially available. (*see, e.g.,* Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. Similarly, arrays and array readers are available, e.g., from Affymetrix, PE Biosystems, and others.

The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

A variety of commercially available peripheral equipment, including, e.g., optical and fluorescent detectors, optical and fluorescent microscopes, plate readers, CCD arrays, phosphorimagers, scintillation counters, phototubes, photodiodes, and the like, and software is available for digitizing, storing and analyzing a digitized video or digitized optical or other assay results, e.g., using PC (Intel x86 or pentium

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chip- compatible DOS™, OS2™ WINDOWST™, WINDOWS NT™ or WINDOW95™ based machines), MACINTOSH™, or UNIX based (e.g., SUN™ work station) computers.

Embodiment in a web site.

The methods described above can be implemented in a localized or distributed computing environment. For example, if a localized computing environment is used, an array comprising a candidate nucleotide library, or diagnostic nucleotide set, is configured in proximity to a detector, which is, in turn, linked to a computational device equipped with user input and output features.

In a distributed environment, the methods can be implemented on a single computer with multiple processors or, alternatively, on multiple computers. The computers can be linked, e.g. through a shared bus, but more commonly, the computer(s) are nodes on a network. The network can be generalized or dedicated, at a local level or distributed over a wide geographic area. In certain embodiments, the computers are components of an intra-net or an internet.

The predictive data corresponding to subject molecular signatures (e.g., expression profiles, and related diagnostic, prognostic, or monitoring results) can be shared by a variety of parties. In particular, such information can be utilized by the subject, the subject's health care practitioner or provider, a company or other institution, or a scientist. An individual subject's data, a subset of the database or the entire database recorded in a computer readable medium can be accessed directly by a user by any method of communication, including, but not limited to, the internet. With appropriate computational devices, integrated systems, communications networks, users at remote locations, as well as users located in proximity to, e.g., at the same physical facility, the database can access the recorded information. Optionally, access to the database can be controlled using unique alphanumeric passwords that provide access to a subset of the data. Such provisions can be used, e.g., to ensure privacy, anonymity, etc.

Typically, a client (e.g., a patient, practitioner, provider, scientist, or the like) executes a Web browser and is linked to a server computer executing a Web server. The Web browser is, for example, a program such as IBM's Web Explorer, Internet explorer, NetScape or Mosaic, or the like. The Web server is typically, but not necessarily, a program such as IBM's HTTP Daemon or other WWW daemon (e.g.,

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LINUX-based forms of the program). The client computer is bi-directionally coupled with the server computer over a line or via a wireless system. In turn, the server computer is bi-directionally coupled with a website (server hosting the website) providing access to software implementing the methods of this invention.

A user of a client connected to the Intranet or Internet may cause the client to request resources that are part of the web site(s) hosting the application(s) providing an implementation of the methods described herein. Server program(s) then process the request to return the specified resources (assuming they are currently available). A standard naming convention has been adopted, known as a Uniform Resource Locator ("URL"). This convention encompasses several types of location names, presently including subclasses such as Hypertext Transport Protocol ("http"), File Transport Protocol ("ftp"), gopher, and Wide Area Information Service ("WAIS"). When a resource is downloaded, it may include the URLs of additional resources. Thus, the user of the client can easily learn of the existence of new resources that he or she had not specifically requested.

Methods of implementing Intranet and/or Intranet embodiments of computational and/or data access processes are well known to those of skill in the art and are documented, e.g., in ACM Press, pp. 383-392; ISO-ANSI, Working Draft, "Information Technology-Database Language SQL", Jim Melton, Editor, International Organization for Standardization and American National Standards Institute, Jul. 1992; ISO Working Draft, "Database Language SQL-Part 2:Foundation (SQL/Foundation)", CD9075-2:199.chi.SQL, Sep. 11, 1997; and Cluer et al. (1992) A General Framework for the Optimization of Object-Oriented Queries, Proc SIGMOD International Conference on Management of Data, San Diego, California, Jun. 2-5, 1992, SIGMOD Record, vol. 21, Issue 2, Jun., 1992; Stonebraker, M., Editor;. Other resources are available, e.g., from Microsoft, IBM, Sun and other software development companies.

Using the tools described above, users of the reagents, methods and database as discovery or diagnostic tools can query a centrally located database with expression and subject data. Each submission of data adds to the sum of expression and subject information in the database. As data is added, a new correlation statistical analysis is automatically run that incorporates the added clinical and expression data. Accordingly, the predictive accuracy and the types of correlations of the recorded molecular signatures increases as the database grows.

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For example, subjects, such as patients, can access the results of the expression analysis of their leukocyte samples and any accrued knowledge regarding the likelihood of the patient's belonging to any specified diagnostic (or prognostic, or monitoring, or risk group), i.e., their expression profiles, and/or molecular signatures. Optionally, subjects can add to the predictive accuracy of the database by providing additional information to the database regarding diagnoses, test results, clinical or other related events that have occurred since the time of the expression profiling. Such information can be provided to the database via any form of communication, including, but not limited to, the internet. Such data can be used to continually define (and redefine) diagnostic groups. For example, if 1000 patients submit data regarding the occurrence of myocardial infarction over the 5 years since their expression profiling, and 300 of these patients report that they have experienced a myocardial infarction and 700 report that they have not, then the 300 patients define a new "group A." As the algorithm is used to continually query and revise the database, a new diagnostic nucleotide set that differentiates groups A and B (i.e., with and without myocardial infarction within a five year period) is identified. This newly defined nucleotide set is then be used (in the manner described above) as a test that predicts the occurrence of myocardial infarction over a five-year period. While submission directly by the patient is exemplified above, any individual with access and authority to submit the relevant data e.g., the patient's physician, a laboratory technician, a health care or study administrator, or the like, can do so.

As will be apparent from the above examples, transmission of information via the internet (or via an intranet) is optionally bi-directional. That is, for example, data regarding expression profiles, subject data, and the like are transmitted via a communication system to the database, while information regarding molecular signatures, predictive analysis, and the like, are transmitted from the database to the user. For example, using appropriate configurations of an integrated system including a microarray comprising a diagnostic nucleotide set, a detector linked to a computational device can directly transmit (locally or from a remote workstation at great distance, e.g., hundreds or thousands of miles distant from the database) expression profiles and a corresponding individual identifier to a central database for analysis according to the methods of the invention. According to, e.g., the algorithms described above, the individual identifier is assigned to one or more diagnostic (or prognostic, or monitoring, etc.) categories. The results of this classification are then

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relayed back, via, e.g., the same mode of communication, to a recipient at the same or different internet (or intranet) address.

Kits

The present invention is optionally provided to a user as a kit. Typically, a kit contains one or more diagnostic nucleotide sets of the invention. Alternatively, the kit contains the candidate nucleotide library of the invention. Most often, the kit contains a diagnostic nucleotide probe set, or other subset of a candidate library, e.g., as a cDNA or antibody microarray packaged in a suitable container. The kit may further comprise, one or more additional reagents, e.g., substrates, labels, primers, for labeling expression products, tubes and/or other accessories, reagents for collecting blood samples, buffers, e.g., erythrocyte lysis buffer, leukocyte lysis buffer, hybridization chambers, cover slips, etc., as well as a software package, e.g., including the statistical methods of the invention, e.g., as described above, and a password and/or account number for accessing the compiled database. The kit optionally further comprises an instruction set or user manual detailing preferred methods of using the diagnostic nucleotide sets in the methods of the invention. Exemplary kits are described in Figure 3.

This invention will be better understood by reference to the following non-limiting Examples:

EXAMPLES

List of Example titles

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Example 14: Identification of diagnostic nucleotide sets for use in diagnosing and treating Restenosis

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Example 16: Identification of diagnostic nucleotide sets for use in diagnosis of rheumatoid arthritis.

Example 17: Identification of diagnostic nucleotide sets for diagnosis of cytomegalovirus

Example 18: Identification of diagnostic nucleotide sets for diagnosis of Epstein Barr Virus

Example 19: Identification of diagnostic nucleotides sets for monitoring response to statin drugs.

Example 20: Probe selection for a 24,000 feature Array.

Example 21: Design of oligonucleotide probes.

Example 22: Production of an array of 8,000 spotted 50 mer oligonucleotides.

Example 23: Amplification, labeling and hybridization of total RNA to an oligonucleotide microarray.

Example 24: Analysis of Human Transplant Patient Mononuclear cell RNA Hybridized to a 24,000 Feature Microarray.

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Examples

Example 1: Generation of subtracted leukocyte candidate nucleotide library

To produce a candidate nucleotide library with representatives from the spectrum of nucleotide sequences that are differentially expressed in leukocytes, subtracted hybridization libraries were produced from the following cell types and conditions:

1. Buffy Coat leukocyte fractions - stimulated with ionomycin and PMA
2. Buffy Coat leukocyte fractions - un-stimulated
3. Peripheral blood mononuclear cells - stimulated with ionomycin and PMA
4. Peripheral blood mononuclear cells - un-stimulated
5. T lymphocytes - stimulated with PMA and ionomycin
6. T lymphocytes - resting

Cells were obtained from multiple individuals to avoid introduction of bias by using only one person as a cell source.

Buffy coats (platelets and leukocytes that are isolated from whole blood) were purchased from Stanford Medical School Blood Center. Four buffy coats were used, each of which was derived from about 350 ml of whole blood from one donor individual. 10 ml of buffy coat sample was drawn from the sample bag using a needle and syringe. 40 ml of Buffer EL (Qiagen) was added per 10 ml of buffy coat to lyse red blood cells. The sample was placed on ice for 15 minutes, and cells were collected by centrifugation at 2000 rpm for 10 minutes. The supernatant was decanted and the cell pellet was re-suspended in leukocyte growth media supplemented with DNase (LGM-3 from Clonetics supplemented with Dnase at a final concentration of 30 U/ml). Cell density was determined using a hemocytometer. Cells were plated in media at a density of 1×10^6 cells/ml in a total volume of 30 ml in a T-75 flask (Corning). Half of the cells were stimulated with ionomycin and phorbol myristate acetate (PMA) at a final concentration of 1 μ g/ml and 62 ng/ml, respectively. Cells were incubated at 37°C and at 5% CO₂ for 3 hours, then cells were scraped off the flask and collected into 50 ml tubes. Stimulated and resting cell populations were kept separate. Cells were centrifuged at 2000 rpm for 10 minutes and the supernatant was removed. Cells were lysed in 6 ml of phenol/guanidine isothiocyanate (Trizol reagent, GibcoBRL), homogenized using a rotary

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homogenizer, and frozen at 80°. Total RNA and mRNA were isolated as described below.

Two frozen vials of 5×10^6 human peripheral blood mononuclear cells (PBMCs) were purchased from Clonetics (catalog number cc-2702). The cells were rapidly thawed in a 37°C water bath and transferred to a 15 ml tube containing 10 ml of leukocyte growth media supplemented with DNase (prepared as described above). Cells were centrifuged at 200g for 10 minutes. The supernatant was removed and the cell pellet was resuspended in LGM-3 media supplemented with DNase. Cell density was determined using a hemocytometer. Cells were plated at a density of 1×10^6 cells/ml in a total volume of 30 ml in a T-75 flask (Corning). Half of the cells were stimulated with ionomycin and PMA at a final concentration of 1 µg/ml and 62 ng/ml, respectively. Cells were incubated at 37°C and at 5% CO₂ for 3 hours, then cells were scraped off the flask and collected into 50 ml tubes. Stimulated and resting cell populations were kept separate. Cells were centrifuged at 2000 rpm and the supernatant was removed. Cells were lysed in 6 ml of phenol/guanidine isothiocyanate solution (TRIZOL reagent, GibcoBRL), homogenized using a rotary homogenizer, and frozen at 80°. Total RNA and mRNA were isolated from these samples using the protocol described below.

45 ml of whole blood was drawn from a peripheral vein of four healthy human subjects into tubes containing anticoagulant. 50 µl RosetteSep (Stem Cell Technologies) cocktail per ml of blood was added, mixed well, and incubated for 20 minutes at room temperature. The mixture was diluted with an equal volume of PBS + 2% fetal bovine serum (FBS) and mixed by inversion. 30 ml of diluted mixture sample was layered on top of 15 ml DML medium (Stem Cell Technologies). The sample tube was centrifuged for 20 minutes at 1200xg at room temperature. The enriched T-lymphocyte cell layer at the plasma : medium interface was removed. Enriched cells were washed with PBS + 2% FBS and centrifuged at 1200 x g. The cell pellet was treated with 5 ml of erythrocyte lysis buffer (EL buffer, Qiagen) for 10 minutes on ice. The sample was centrifuged for 5 min at 1200g. Cells were plated at a density of 1×10^6 cells/ml in a total volume of 30 ml in a T-75 flask (Corning). Half of the cells were stimulated with ionomycin and PMA at a final concentration of 1 µg/ml and 62 ng/ml, respectively. Cells were incubated at 37°C and at 5% CO₂ for 3 hours, then cells were scraped off the flask and collected into 50 ml tubes. Stimulated and resting cell populations were kept separate. Cells were centrifuged at 2000 rpm

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and the supernatant was removed. Cells were lysed in 6 ml of phenol/guanidine isothiocyanate solution (TRIZOL reagent, GibcoBRL), homogenized using a rotary homogenizer, and frozen at 80°. Total RNA and mRNA were isolated as described below.

Total RNA and mRNA were isolated using the following procedure: the homogenized samples were thawed and mixed by vortexing. Samples were lysed in a 1:0.2 mixture of Trizol and chloroform, respectively. For some samples, 6 ml of Trizol-chloroform was added. Variable amounts of Trizol-chloroform was added to other samples. Following lysis, samples were centrifuged at 3000 g for 15 min at 4°C. The aqueous layer was removed into a clean tube and 4 volumes of Buffer RLT (Qiagen) was added for every volume of aqueous layer. The samples were mixed thoroughly and total RNA was prepared from the sample by following the Qiagen Rneasy midi protocol for RNA cleanup (October 1999 protocol, Qiagen). For the final step, the RNA was eluted from the column twice with 250 µl Rnase-free water. Total RNA was quantified using a spectrophotometer. Isolation of mRNA from total RNA sample was done using The Oligotex mRNA isolation protocol (Qiagen) was used to isolate mRNA from total RNA, according to the manufacturer's instructions (Qiagen, 7/99 version). mRNA was quantified by spectrophotometry.

Subtracted cDNA libraries were prepared using Clontech's PCR-Select cDNA Subtraction Kit (protocol number PT-1117-1) as described in the manufacturer's protocol. The protocol calls for two sources of RNA per library, designated "Driver" and "Tester." The following 6 libraries were made:

<u>Library</u>	<u>Driver RNA</u>	<u>Tester RNA</u>
Buffy Coat Stimulated	Un-stimulated Buffy Coat	Stimulated Buffy Coat
Buffy Coat Resting	Stimulated Buffy Coat	Un-stimulated Buffy Coat
PBMC Stimulated	Un-stimulated PBMCs	Stimulated PBMCs
PBMC Resting	Stimulated PBMCs	Un-stimulated PBMCs
T-cell Stimulated	Un-stimulated T-cells	Stimulated T-cells
T-cell Resting	Stimulated T-cells	Un-stimulated T-cells

The Clontech protocol results in the PCR amplification of cDNA products. The PCR products of the subtraction protocol were ligated to the pGEM T-easy bacterial vector as described by the vector manufacturer (Promega 6/99 version). Ligated vector was transformed into competent bacteria using well-known techniques,

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plated, and individual clones are picked, grown and stored as a glycerol stock at –80°C. Plasmid DNA was isolated from these bacteria by standard techniques and used for sequence analysis of the insert. Unique cDNA sequences were searched in the Unigene database (build 133), and Unigene cluster numbers were identified that corresponded to the DNA sequence of the cDNA. Unigene cluster numbers were recorded in an Excel spreadsheet.

Example 2: Identification of nucleotide sequences for candidate library using data mining techniques

Existing and publicly available gene sequence databases were used to identify candidate nucleotide sequences for leukocyte expression profiling. Genes and nucleotide sequences with specific expression in leukocytes, for example, lineage specific markers, or known differential expression in resting or activated leukocytes were identified. Such nucleotide sequences are used in a leukocyte candidate nucleotide library, alone or in combination with nucleotide sequences isolated through cDNA library construction, as described above.

Leukocyte candidate nucleotide sequences were identified using three primary methods. First, the publically accessible publication database PubMed was searched to identify nucleotide sequences with known specific or differential expression in leukocytes. Nucleotide sequences were identified that have been demonstrated to have differential expression in peripheral blood leukocytes between subjects with and without particular disease(s) selected from Table 1. Additionally, genes and gene sequences that were known to be specific or selective for leukocytes or sub-populations of leukocytes were identified in this way.

Next, two publicly available databases of DNA sequences, Unigene (<http://www.ncbi.nlm.nih.gov/UniGene/>) and BodyMap (<http://bodymap.ims.u-tokyo.ac.jp/>), were searched for sequenced DNA clones that showed specificity to leukocyte lineages, or subsets of leukocytes, or resting or activated leukocytes.

The human Unigene database (build 133) was used to identify leukocyte candidate nucleotide sequences that were likely to be highly or exclusively expressed in leukocytes. We used the Library Differential Display utility of Unigene (<http://www.ncbi.nlm.nih.gov/UniGene/info/ddd.html>), which uses statistical methods (The Fisher Exact Test) to identify nucleotide sequences that have relative specificity

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for a chosen library or group of libraries relative to each other. We compared the following human libraries from Unigene release 133:

546 NCI_CGAP_HSC1 (399)
 848 Human_mRNA_from_cd34+_stem_cells (122)
 105 CD34+DIRECTIONAL (150)
 3587 KRIBB_Human_CD4_intrathymic_T-cell_cDNA_library (134)
 3586 KRIBB_Human_DP_intrathymic_T-cell_cDNA_library (179)
 3585 KRIBB_Human_TN_intrathymic_T-cell_cDNA_library (127)
 3586 323 Activated_T-cells_I (740)
 376 Activated_T-cells_XX (1727)
 327 Monocytes,_stimulated_II (110)
 824 Proliferating_Erythroid_Cells_(LCB:ad_library) (665)
 825 429 Macrophage_II (105)
 387 Macrophage_I (137)
 669 NCI_CGAP_CLL1 (11626)
 129 Human_White_blood_cells (922)
 1400 NIH_MGC_2 (422)
 55 Human_promyelocyte (1220)
 1010 NCI_CGAP_CML1 (2541)
 2217 NCI_CGAP_Sub7 (218)
 1395 NCI_CGAP_Sub6 (2764)
 4874 NIH_MGC_48 (2524)

BodyMap, like Unigene, contains cell-specific libraries that contain potentially useful information about genes that may serve as lineage-specific or leukocyte specific markers (Okubo et al. 1992). We compared three leukocyte specific libraries, Granulocyte, CD4 T cell, and CD8 T cell, with the other libraries. Nucleotide sequences that were found in one or more of the leukocyte-specific libraries, but absent in the others, were identified. Clones that were found exclusively in one of the three leukocyte libraries were also included in a list of nucleotide sequences that could serve as lineage-specific markers.

Next, the sequence of the nucleotide sequences identified in PubMed or BodyMap were searched in Unigene (version 133), and a human Unigene cluster number was identified for each nucleotide sequence. The cluster number was

recorded in a Microsoft Excel™ spreadsheet, and a non-redundant list of these clones was made by sorting the clones by UniGene number, and removing all redundant clones using Microsoft Excel™ tools. The non-redundant list of UniGene cluster numbers was then compared to the UniGene cluster numbers of the cDNAs identified using differential cDNA hybridization, as described above in Example 1 (listed in Table 3 and the sequence listing). Only UniGene clusters that were not contained in the cDNA libraries were retained. UniGene clusters corresponding to 1911 candidate nucleotide sequences for leukocyte expression profiling were identified in this way and are listed in Table 3 and the sequence listing.

DNA clones corresponding to each UniGene cluster number are obtained in a variety of ways. First, a cDNA clone with identical sequence to part of, or all of the identified UniGene cluster is bought from a commercial vendor or obtained from the IMAGE consortium (<http://image.llnl.gov/>, the Integrated Molecular Analysis of Genomes and their Expression). Alternatively, PCR primers are designed to amplify and clone any portion of the nucleotide sequence from cDNA or genomic DNA using well-known techniques. Alternatively, the sequences of the identified UniGene clusters are used to design and synthesize oligonucleotide probes for use in microarray based expression profiling.

Example 3: DNA Sequencing and Processing of raw sequence data.

Clones of differentially expressed cDNAs (identified by subtractive hybridization, described above) were sequenced on an MJ Research BaseStation™ slab gel based fluorescent detection system, using BigDye™ (Applied Biosystems, Foster City, CA) terminator chemistry was used (Heiner et al., Genome Res 1998 May;8(5):557-61).

The fluorescent profiles were analyzed using the Phred sequence analysis program (Ewing et al, (1998), Genome Research 8: 175-185). Analysis of each clone results in a one pass nucleotide sequence and a quality file containing a number for each base pair with a score based on the probability that the determined base is correct. Each sequence files and its respective quality files were initially combined into single fasta format (Pearson, WR. Methods Mol Biol. 2000;132:185-219), multi-sequence file with the appropriate labels for each clone in the headers for subsequent automated analysis.

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Initially, known sequences were analyzed by pair wise similarity searching using the `blastn` option of the `blastall` program obtained from the National Center for Biological Information, National Library of Medicine, National Institutes of Health (NCBI) to determine the quality score that produced accurate matching (Altschul SF, et al. *J Mol Biol.* 1990 Oct 5;215(3):403-10.). Empirically, it was determined that a raw score of 8 was the minimum that contained useful information. Using a sliding window average for 16 base pairs, an average score was determined. The sequence was removed (trimmed) when the average score fell below 8. Maximum reads were 950 nucleotides long.

Next, the sequences were compared by similarity matching against a database file containing the flanking vector sequences used to clone the cDNA, using the `blastall` program with the `blastn` option. All regions of vector similarity were removed, or "trimmed" from the sequences of the clones using scripts in the GAWK programming language, a variation of AWK (Aho AV et al, *The Awk Programming Language* (Addison-Wesley, Reading MA, 1988); Robbins, AD, "Effective AWK Programming" (Free Software Foundation, Boston MA, 1997). It was found that the first 45 base pairs of all the sequences were related to vector; these sequences were also trimmed and thus removed from consideration. The remaining sequences were then compared against the NCBI vector database (Kitts, P.A. et al. National Center for Biological Information, National Library of Medicine, National Institutes of Health, Manuscript in preparation (2001) using `blastall` with the `blastn` option. Any vector sequences that were found were removed from the sequences.

Messenger RNA contains repetitive elements that are found in genomic DNA. These repetitive elements lead to false positive results in similarity searches of query mRNA sequences versus known mRNA and EST databases. Additionally, regions of low information content (long runs of the same nucleotide, for example) also result in false positive results. These regions were masked using the program RepeatMasker2 found at <http://repeatmasker.genome.washington.edu> (Smit, AFA & Green, P "RepeatMasker" at <http://ftp.genome.washington.edu/RM/RepeatMasker.html>). The trimmed and masked files were then subjected to further sequence analysis.

Example 4: Further sequence analysis of novel nucleotide sequences identified by subtractive hybridization screening

cDNA sequences were further characterized using BLAST analysis. The BLASTN program was used to compare the sequence of the fragment to the UniGene, dbEST, and nr databases at NCBI (GenBank release 123.0; see Table 5). In the BLAST algorithm, the expect value for an alignment is used as the measure of its significance. First, the cDNA sequences were compared to sequences in Unigene (<http://www.ncbi.nlm.nih.gov/UniGene>). If no alignments were found with an expect value less than 10^{-25} , the sequence was compared to the sequences in the dbEST database using BLASTN. If no alignments were found with an expect value less than 10^{-25} , the sequence was compared to sequences in the nr database.

The BLAST analysis produced the following categories of results: a) a significant match to a known or predicted human gene, b) a significant match to a nonhuman DNA sequence, such as vector DNA or *E. coli* DNA, c) a significant match to an unidentified GenBank entry (a sequence not previously identified or predicted to be an expressed sequence or a gene), such as a cDNA clone, mRNA, or cosmid, or d) no significant alignments. If a match to a known or predicted human gene was found, analysis of the known or predicted protein product was performed as described below. If a match to an unidentified GenBank entry was found, or if no significant alignments were found, the sequence was searched against all known sequences in the human genome database (<http://www.ncbi.nlm.nih.gov/genome/seq/page.cgi?F=HsBlast.html&&ORG=Hs>, see Table 5).

If many unknown sequences were to be analyzed with BLASTN, the clustering algorithm CAP2 (Contig Assembly Program, version 2) was used to cluster them into longer, contiguous sequences before performing a BLAST search of the human genome. Sequences that can be grouped into contigs are likely to be cDNA from expressed genes rather than vector DNA, *E. coli* DNA or human chromosomal DNA from a noncoding region, any of which could have been incorporated into the library. Clustered sequences provide a longer query sequence for database comparisons with BLASTN, increasing the probability of finding a significant match to a known gene. When a significant alignment was found, further analysis of the putative gene was performed, as described below. Otherwise, the sequence of the

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original cDNA fragment or the CAP2 contig is used to design a probe for expression analysis and further approaches are taken to identify the gene or predicted gene that corresponds to the cDNA sequence, including similarity searches of other databases, molecular cloning, and Rapid Amplification of cDNA Ends (RACE).

In some cases, the process of analyzing many unknown sequences with BLASTN was automated by using the BLAST network-client program blastcl3, which was downloaded from <ftp://ncbi.nlm.nih.gov/blast/network/netblast>.

When a cDNA sequence aligned to the sequence of one or more chromosomes, a large piece of the genomic region around the loci was used to predict the gene containing the cDNA. To do this, the contig corresponding to the mapped locus, as assembled by the RefSeq project at NCBI, was downloaded and cropped to include the region of alignment plus 100,000 bases preceding it and 100,000 bases following it on the chromosome. The result was a segment 200 kb in length, plus the length of the alignment. This segment, designated a putative gene, was analyzed using an exon prediction algorithm to determine whether the alignment area of the unknown sequence was contained within a region predicted to be transcribed (see Table 6).

This putative gene was characterized as follows: all of the exons comprising the putative gene and the introns between them were taken as a unit by noting the residue numbers on the 200kb+ segment that correspond to the first base of the first exon and the last base of the last exon, as given in the data returned by the exon prediction algorithm. The truncated sequence was compared to the UniGene, dbEST, and nr databases to search for alignments missed by searching with the initial fragment.

The predicted amino acid sequence of the gene was also analyzed. The peptide sequence of the gene predicted from the exons was used in conjunction with numerous software tools for protein analysis (see Table 7). These were used to classify or identify the peptide based on similarities to known proteins, as well as to predict physical, chemical, and biological properties of the peptides, including secondary and tertiary structure, flexibility, hydrophobicity, antigenicity (hydrophilicity), common domains and motifs, and localization within the cell or tissues. The peptide sequence was compared to protein databases, including SWISS-PROT, TrEMBL, GenPept, PDB, PIR, PROSITE, ProDom, PROSITE, Blocks,

PRINTS, and Pfam, using BLASTP and other algorithms to determine similarities to known proteins or protein subunits.

Example 5: Further sequence analysis of novel Clone 596H6

The sequence of clone 596H6 is provided below:

ACTATATTTA	GGCACCCTG	CCATAAACTA	CCAAAAA	AATGTAATTC	50
CTAGAAGCTG	TGAAGAATAG	TAGTGTAGCT	AAGCACGGTG	TGTGGACAGT	100
GGGACATCTG	CCACCTGCAG	TAGGTCTCTG	CACTCCCAA	AGCAAATTAC	150
ATTGGCTTGA	ACTTCAGTAT	GCCCGGTTCC	ACCCTCCAGA	AACTTTTGTG	200
TTCTTTGTAT	AGAATTTAGG	AACTTCTGAG	GGCCACAAAT	ACACACATTA	250
AAAAAGGTAG	AATTTTTGAA	GATAAGATTC	TTCTAAAAA	GCTTCCCAAT	300
GCTTGAGTAG	AAAGTATCAG	TAGAGGTATC	AAGGGAGGAG	AGACTAGGTG	350
ACCACTAAAC	TCCTTCAGAC	TCTTAAAT	ACGATTCTTT	TCTCAAAGGG	400
GAAGAACGTC	AGTGCAGCGA	TCCCTTCACC	TTTAGCTAAA	GAATTGGACT	450
GTGCTGCTCA	AAATAAAGAT	CAGTTGGAGG	TANGATGTCC	AAGACTGAAG	500
GTAAAGGACT	AGTGCAAAC	GAAAGTGATG	GGGAAACAGA	CCTACGTATG	550
GAAGCCATGT	AGTGTTCTTC	ACAGGCTGCT	GTTGACTGAA	ATTCCTATCC	600
TCAAATTACT	CTAGACTGAA	GCTGCTTCCC	TTCAGTGAGC	AGCCTCTCCT	650
TCCAAGATTC	TGGAAAGCAC	ACCTGACTCC	AAACAAAGAC	TTAGAGCCCT	700
GTGTCAGTGC	TGCTGCTGCT	TTTACCAGAT	TCTCTAACCT	TCCGGGTAGA	750

AGAG (SEQ ID NO:
8767)

This sequence was used as input for a series of BLASTN searches. First, it was used to search the UniGene database, build 132 (<http://www.ncbi.nlm.nih.gov/BLAST/>). No alignments were found with an expect value less than the threshold value of 10^{-25} . A BLASTN search of the database dbEST, release 041001, was then performed on the sequence and 21 alignments were found (<http://www.ncbi.nlm.nih.gov/BLAST/>). Ten of these had expect values less than 10^{-25} , but all were matches to unidentified cDNA clones. Next, the sequence was used to run a BLASTN search of the nr database, release 123.0. No significant alignment to any sequence in nr was found. Finally, a BLASTN search of the human genome was performed on the sequence (<http://www.ncbi.nlm.nih.gov/genome/seq/page.cgi?F=HsBlast.html&&ORG=Hs>).

A single alignment to the genome was found on contig NT_004698.3 ($e=0.0$). The region of alignment on the contig was from base 1,821,298 to base 1,822,054,

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and this region was found to be mapped to chromosome 1, from base 105,552,694 to base 105,553,450. The sequence containing the aligned region, plus 100 kilobases on each side of the aligned region, was downloaded. Specifically, the sequence of chromosome 1 from base 105,452,694 to 105,653,450 was downloaded (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/seq_reg.cgi?chr=1&from=105452694&to=105653450).

This 200,757 bp segment of the chromosome was used to predict exons and their peptide products as follows. The sequence was used as input for the Genscan algorithm (<http://genes.mit.edu/GENSCAN.html>), using the following Genscan settings:

Organism: vertebrate

Suboptimal exon cutoff: 1.00 (no suboptimal exons)

Print options: Predicted CDS and peptides

The region matching the sequence of clone 596H6 was known to span base numbers 100,001 to 100,757 of the input sequence. An exon was predicted by the algorithm, with a probability of 0.695, covering bases 100,601 to 101,094 (designated exon 4.14 of the fourth predicted gene). This exon was part of a predicted cistron that is 24,195 bp in length. The sequence corresponding to the cistron was noted and saved separately from the 200,757 bp segment. BLASTN searches of the Unigene, dbEST, and nr databases were performed on it.

At least 100 significant alignments to various regions of the sequence were found in the dbEST database, although most appeared to be redundant representations of a few exons. All matches were to unnamed cDNAs and mRNAs (unnamed cDNAs and mRNAs are cDNAs and mRNAs not previously identified, or shown to correspond to a known or predicted human gene) from various tissue types. Most aligned to a single region on the sequence and spanned 500 bp or less, but several consisted of five or six regions separated by gaps, suggesting the locations of exons in the gene. Several significant matches to entries in the UniGene database were found, as well, even after masking low-complexity regions and short repeats in the sequence. All matches were to unnamed cDNA clones.

At least 100 significant alignments were found in the nr database, as well. A similarity to hypothetical protein FLJ22457 (UniGene cluster Hs.238707) was found ($e=0.0$). The cDNA of this predicted protein has been isolated from B lymphocytes

(<http://www.ncbi.nlm.nih.gov/entrez/viewer.cgi?save=0&cmd=&cfm=on&f=1&view=gp&txt=0&val=13637988>).

Other significant alignments were to unnamed cDNAs and mRNAs.

Using Genscan, the following 730 residue peptide sequence was predicted from the putative gene:

MDGLGRRRLRA	SLRLKRGHGG	HWRLNEMPYM	KHEFDGGPPQ	DNSGEALKEP	5
ERAQEHSLPN	FAGGQHFFEY	LLVSLKKKR	SEDDYEPIIT	YQFPKRENLL	1
RGQEEEEERL	LKAIPFCFP	DGNEWASLTE	YPSLSCKTPG	LLAALVVEKA	1
QPRTCCHASA	PSAAPQARGP	DAPSPAAGQA	LPAGPGPRLP	KVYCIISCIG	2
CFGLFSKILD	EVEKRHQISM	AVIYPFMQGL	REAAFPAPGK	TVTLKSFIPD	2
SGTEFISLTR	PLDSHLEHVD	FSSLLHCLSF	EQILQIFASA	VLERKIIFLA	3
EGLREEEKDV	RDSTEVRGAG	ECHGFQRKGN	LGKQWGLCVE	DSVKMGDNQR	3
GTSCSTLSQC	IHAAAALLYP	FSWAHTYIPV	VPESLLATVC	CPTPFMVG VQ	4
MRFQQEVMDS	PMEEIQPQAE	IKTVNPLGVY	EERGPEKASL	CLFQVLLVNL	4
CEGTFLMSVG	DEKDILPPKL	QDDILDSLQ	GINELKTAEQ	INEHVSGPFV	5
QFFVKIVGHY	ASYIKREANG	QGHFQERSFC	KALTSKTNRR	FVKKFVKTQL	5
FSLFIQAEK	SKNPPAEVTQ	VGNSSTCVVD	TWLEAAATAL	SHHYNIFNTE	6
HTLWSKGSAS	LHEVCGHVRT	RVKRKILFLY	VSLAFTMGKS	IFLVENKAMN	6
MTIKWTTSGR	PGHGDMFGVI	ESWGAAALLL	LTGRVRDTGK	SSSSTGHRAS	7
KSLVWSQVCF	PESWEERLLT	EGKQLQSRVI	SEQ ID NO:8768		

Multiple analyses were performed using this prediction. First, a pairwise comparison of the sequence above and the sequence of FLJ22457, the hypothetical protein mentioned above, using BLASTP version 2.1.2

(<http://ncbi.nlm.nih.gov/BLAST/>), resulted in a match with an expect value of 0.0.

The peptide sequence predicted from clone 596H6 was longer and 19% of the region of alignment between the two resulted from gaps in hypothetical protein FLJ22457. The cause of the discrepancy might be alternative mRNA splicing, alternative post-translational processing, or differences in the peptide-predicting algorithms used to create the two sequences, but the homology between the two is significant.

BLASTP and TBLASTN were also used to search for sequence similarities in the SWISS-PROT, TrEMBL, GenBank Translated, and PDB databases. Matches to several proteins were found, among them a tumor cell suppression protein, HTS1. No

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matches aligned to the full length of the peptide sequence, however, suggesting that similarity is limited to a few regions of the peptide.

TBLASTN produced matches to several proteins – both identified and theoretical – but again, no matches aligned to the full length of the peptide sequence. The best alignment was to the same hypothetical protein found in GenBank before (FLJ22457).

To discover similarities to protein families, comparisons of the domains (described above) were carried out using the Pfam and Blocks databases. A search of the Pfam database identified two regions of the peptide domains as belonging the DENN protein family ($e=2.1 \times 10^{-33}$). The human DENN protein possesses an RGD cellular adhesion motif and a leucine-zipper-like motif associated with protein dimerization, and shows partial homology to the receptor binding domain of tumor necrosis factor alpha. DENN is virtually identical to MADD, a human MAP kinase-activating death domain protein that interacts with type I tumor necrosis factor receptor ([http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-id+fS5n1GQsHf+-e+\[INTERPRO:IPR001194'\]](http://srs.ebi.ac.uk/srs6bin/cgi-bin/wgetz?-id+fS5n1GQsHf+-e+[INTERPRO:IPR001194'])). The search of the Blocks database also revealed similarities between regions of the peptide sequence and known protein groups, but none with a satisfactory degree of confidence. In the Blocks scoring system, scores over 1,100 are likely to be relevant. The highest score of any match to the predicted peptide was 1,058.

The Prosite, ProDom, PRINTS databases (all publicly available) were used to conduct further domain and motif analysis. The Prosite search generated many recognized protein domains. A BLASTP search was performed to identify areas of similarity between the protein query sequence and PRINTS, a protein database of protein fingerprints, groups of motifs that together form a characteristic signature of a protein family. In this case, no groups were found to align closely to any section of the submitted sequence. The same was true when the ProDom database was searched with BLASTP.

A prediction of protein structure was done by performing a BLAST search of the sequence against PDB, a database in which every member has tertiary structure information. No significant alignments were found by this method. Secondary and super-secondary structure was examined using the Garnier algorithm. Although it is only considered to be 60-65% accurate, the algorithm provided information on the locations and lengths of alpha-helices, beta-sheets, turns and coils.

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The antigenicity of the predicted peptide was modeled by graphing hydrophilicity vs. amino acid number. This produced a visual representation of trends in hydrophilicity along the sequence. Many locations in the sequence showed antigenicity and five sites had antigenicity greater than 2. This information can be used in the design of affinity reagents to the protein.

Membrane-spanning regions were predicted by graphing hydrophobicity vs. amino acid number. Thirteen regions were found to be somewhat hydrophobic. The algorithm TMPred predicted a model with 6 strong transmembrane helices (http://www.ch.embnet.org/software/TMPRED_form.html).

NNPSL is a neural network algorithm developed by the Sanger Center. It uses amino acid composition and sequence to predict cellular location. For the peptide sequence submitted, its first choice was mitochondrial (51.1% expected accuracy). Its second choice was cytoplasmic (91.4% expected accuracy).

Example 6: Further sequence analysis of novel Clone 486E11

The sequence of clone 486E11 is provided below:

TAAAAGCAGG	CTGTGCACTA	GGGACCTAGT	GACCTTACTA	GAAAAAACTC	5
AAATTCTCTG	AGCCACAAGT	CCTCATGGGC	AAAATGTAGA	TACCACCACC	1
TAACCCTGCC	AATTCCTAT	CATTGTGACT	ATCAAATTAA	ACCACAGGCA	1
GGAAGTTGCC	TTGAAAACCT	TTTATAGTGT	ATATTACTGT	TCACATAGAT	2
NAGCAATTAA	CTTTACATAT	ACCCGTTTTT	AAAAGATCAG	TCCTGTGATT	2
AAAAGTCTGG	CTGCCCTAAT	TCACTTCGAT	TATACATTAG	GTAAAGCCA	3
TATAAAAGAG	GCACTACGTC	TTCGGAGAGA	TGAATGGATA	TTACAAGCAG	3
TAATGTTGGC	TTTGGAATAT	ACACATAATG	TCCACTTGAC	CTCATCTATT	4
TGACACAAAA	TGTAAACTAA	ATTATGAGCA	TCATTAGATA	CCTTGGCCTT	4
TTCAAATCAC	ACAGGGTCCT	AGATCTNNNN	NNNNNNNNNN	NNNNNNNNNN	5
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNAC	TTTGGGATTG	5
CTATATCTTT	GTCAGCTGTC	AACTTCAGTG	TTTTCAGGTT	AAATTCTATC	6
CATAGTCATC	CCAATATACC	TGCTTTAGAT	GATACAACCT	TCAAAGATC	6
CGCTCTTCCT	CGTAAAAAGT	GGAG	SEQ ID NO: 8769		

The BLASTN program was used to compare the sequence to the UniGene and dbEST databases. No significant alignments were found in either. It was then searched against the nr database and only alignments to unnamed genomic DNA clones were found.

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CAP2 was used to cluster a group of unknowns, including clone 486E11. The sequence for 486E11 was found to overlap others. These formed a contig of 1,010 residues, which is shown below:

CGGACAGGTA	CCTAAAAGCA	GGCTGTGCAC	TAGGGACCTA	GTGACCTTAC	5
TAGAAAAAAC	TCAAATTCTC	TGAGCCACAA	GTCCTCATGG	GCAAAATGTA	1
GATACCACCA	CCTAACCCCTG	CCAATTTCCCT	ATCATTGTGA	CTATCAAATT	1
AAACCACAGG	CAGGAAGTTG	CCTTGAAAAC	TTTTTATAGT	GTATATTACT	2
GTTACATAG	ATNAGCAATT	AACTTTACAT	ATACCCGTTT	TTAAAAGATC	2
AGTCCTGTGA	TTAAAAGTCT	GGCTGCCCTA	ATTCACTTCG	ATTATACATT	3
AGGTAAAGC	CATATAAAAG	AGGCACTACG	TCTTCGGAGA	GATGAATGGA	3
TATTACAAGC	AGTAATTTTG	GCTTTGGAAT	ATACACATAA	TGTCCACTTG	4
ACCTCATCTA	TTTGACACAA	AATGTAAACT	AAATTATGAG	CATCATTAGA	4
TACCTTGGGC	CTTTTCAAAT	CACACAGGGT	CCTAGATCTG	NNNNNNNNNN	5
NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	NNNNNNNNNN	5
NACTTTGGAT	TCTTATATCT	TTGTCAGCTG	TCAACTTCAG	TGTTTTTCAGG	6
NTAAATTCTA	TCCATAGTCA	TCCCAATATA	CCTGCTTTAG	ATGATACAAA	6
CTTCAAAAGA	TCCGGCTCTC	CCTCGTAAAA	CGTGGAGGAC	AGACATCAAG	7
GGGGTTTTCT	GAGTAAAGAA	AGGCAACCGC	TCGGCAAAAA	CTCACCCCTGG	7
CACAACAGGA	NCGAATATAT	ACAGACGCTG	ATTGAGCGTT	TTGCTCCATC	8
TTCACTTCTG	TTAAATGAAG	ACATTGATAT	CTAAAATGCT	ATGAGTCTAA	8
CTTTGTAAAA	TTAAAATAGA	TTTGTAGTTA	TTTTTCAAAA	TGAAATCGAA	9
AAGATACAAG	TTTTGAAGGC	AGTCTCTTTT	TCCACCCTGC	CCCTCTAGTG	9
TGTTTTACAC	ACTTCTCTGG	CCACTCCAAC	AGGGAAGCTG	GTCCAGGGCC	1
ATTATACAGG	SEQ ID NO: 8832				

The sequence of the CAP2 contig was used in a BLAST search of the human genome. 934 out of 1,010 residues aligned to a region of chromosome 21. A gap of 61 residues divided the aligned region into two smaller fragments. The sequence of this region, plus 100 kilobases on each side of it, was downloaded and analyzed using the Genscan site at MIT (<http://genes.mit.edu/GENSCAN.html>), with the following settings:

Organism: vertebrate

Suboptimal exon cutoff: 1.00 (no suboptimal exons)

Print options: Predicted CDS and peptides

The fragment was found to fall within one of several predicted genes in the chromosome region. The bases corresponding to the predicted gene, including its predicted introns, were saved as a separate file and used to search GenBank again with BLASTN to find any ESTs or UniGene clusters identified by portions of the sequence not included in the original unknown fragment. The nr database contained no significant matches. At least 100 significant matches to various parts of the predicted gene were found in the dbEST database, but all of them were to unnamed cDNA clones. Comparison to UniGene produced fewer significant matches, but all matches were to unnamed cDNAs.

The peptide sequence predicted by Genscan was also saved. Multiple types of analyses were performed on it using the resources mentioned in Table 3. BLASTP and TBLASTN were used to search the TrEMBL protein database (<http://www.expasy.ch/sprot/>) and the GenBank nr database (<http://www.ncbi.nlm.nih.gov/BLAST/>), which includes data from the SwissProt, PIR, PRF, and PDB databases. No significant matches were found in any of these, so no gene identity or tertiary structure was discovered.

The peptide sequence was also searched for similarity to known domains and motifs using BLASTP with the Prosite, Blocks, Pfam, and ProDom databases. The searches produced no significant alignments to known domains. BLASTP comparison to the PRINTS database produced an alignment to the P450 protein family, but with a low probability of accuracy ($e=6.9$).

Two methods were used to predict secondary structure – the Garnier/Osguthorpe/Robson model and the Chou-Fasman model. The two methods differed somewhat in their results, but both produced representations of the peptide sequence with helical and sheet regions and locations of turns.

Antigenicity was plotted as a graph with amino acid number in the sequence on the x-axis and hydrophilicity on the y-axis. Several areas of antigenicity were observed, but only one with antigenicity greater than 2. Hydrophobicity was plotted in the same way. Only one region, from approximately residue 135 to residue 150, had notable hydrophobicity. TMpred, accessed through ExPASy, was used to predict transmembrane helices. No regions of the peptide sequence were predicted with reasonable confidence to be membrane-spanning helices.

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NNPSL predicted that the putative protein would be found either in the nucleus (expected prediction accuracy = 51.1%) or secreted from the cell (expected prediction accuracy = 91.4%).

Example 7: Preparation of a leukocyte cDNA array comprising a candidate gene library

Candidate genes and gene sequences for leukocyte expression profiling were identified through methods described elsewhere in this document. Candidate genes are used to obtain or design probes for peripheral leukocyte expression profiling in a variety of ways.

A cDNA microarray carrying 384 probes was constructed using sequences selected from the cDNA libraries described in example 1. cDNAs were selected from T-cell libraries, PBMC libraries and buffy coat libraries. A listing of the cDNA fragments used is given in Table 8.

96-Well PCR

Plasmids were isolated in 96-well format and PCR was performed in 96-well format. A master mix was made that contain the reaction buffer, dNTPs, forward and reverse primer and DNA polymerase was made. 99 ul of the master mix was aliquoted into 96-well plate. 1 ul of plasmid (1-2 ng/ul) of plasmid was added to the plate. The final reaction concentration was 10 mM Tris pH 8.3, 3.5 mM MgCl₂, 25 mM KCl, 0.4 mM dNTPs, 0.4 uM M13 forward primer, 0.4 M13 reverse primer, and 10 U of Taq Gold (Applied Biosystems). The PCR conditions were:

- Step 1 95C for 10 min
- Step 2 95C for 15 sec
- Step 3 56C for 30 sec
- Step 4 72C for 2 min 15 seconds
- Step 5 go to Step 2 39 times
- Step 6 72C for 10 minutes
- Step 7 4C for ever.

PCR Purification

PCR purification was done in a 96-well format. The ArrayIt (Telechem International, Inc.) PCR purification kit was used and the provided protocol was followed without modification. Before the sample was evaporated to dryness, the

concentration of PCR products was determined using a spectrophotometer. After evaporation, the samples were re-suspended in 1x Micro Spotting Solution (ArrayIt) so that the majority of the samples were between 0.2-1.0 ug/ul.

Array Fabrication

Spotted cDNA microarrays were then made from these PCR products by ArrayIt using their protocols (http://arrayit.com/Custom_Microarrays/Flex-Chips/flex-chips.html). Each fragment was spotted 3 times onto each array.

Candidate genes and gene sequences for leukocyte expression profiling were identified through methods described elsewhere in this document. Those candidate genes are used for peripheral leukocyte expression profiling. The candidate libraries can be used to obtain or design probes for expression profiling in a variety of ways.

Oligonucleotide probes are also prepared using the DNA sequence information for the candidate genes identified by differential hybridization screening (listed in Table 3 and the sequence listing) and/or the sequence information for the genes identified by database mining (listed in Table 2) is used to design complementary oligonucleotide probes. Oligo probes are designed on a contract basis by various companies (for example, Compugen, Mergen, Affymetrix, Telechem), or designed from the candidate sequences using a variety of parameters and algorithms as indicated at <http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi>. Briefly, the length of the oligonucleotide to be synthesized is determined, preferably greater than 18 nucleotides, generally 18-24 nucleotides, 24-70 nucleotides and, in some circumstances, more than 70 nucleotides. The sequence analysis algorithms and tools described above are applied to the sequences to mask repetitive elements, vector sequences and low complexity sequences. Oligonucleotides are selected that are specific to the candidate nucleotide sequence (based on a Blast n search of the oligonucleotide sequence in question against gene sequences databases, such as the Human Genome Sequence, UniGene, dbEST or the non-redundant database at NCBI), and have <50% G content and 25-70% G+C content. Desired oligonucleotides are synthesized using well-known methods and apparatus, or ordered from a company (for example Sigma). Oligonucleotides are spotted onto microarrays. Alternatively, oligonucleotides are synthesized directly on the array surface, using a variety of techniques (Hughes et al. 2001, Yershov et al. 1996, Lockhart et al 1996).

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Example 8: Preparation of RNA from mononuclear cells for expression profiling

Blood was isolated from the subject for leukocyte expression profiling using the following methods:

Two tubes were drawn per patient. Blood was drawn from either a standard peripheral venous blood draw or directly from a large-bore intra-arterial or intravenous catheter inserted in the femoral artery, femoral vein, subclavian vein or internal jugular vein. Care was taken to avoid sample contamination with heparin from the intravascular catheters, as heparin can interfere with subsequent RNA reactions.

For each tube, 8 ml of whole blood was drawn into a tube (CPT, Becton-Dickinson order #362753) containing the anticoagulant Citrate, 25°C density gradient solution (e.g. Ficoll, Percoll) and a polyester gel barrier that upon centrifugation was permeable to RBCs and granulocytes but not to mononuclear cells. The tube was inverted several times to mix the blood with the anticoagulant. The tubes were centrifuged at 1750xg in a swing-out rotor at room temperature for 20 minutes. The tubes were removed from the centrifuge and inverted 5-10 times to mix the plasma with the mononuclear cells, while trapping the RBCs and the granulocytes beneath the gel barrier. The plasma/mononuclear cell mix was decanted into a 15ml tube and 5ml of phosphate-buffered saline (PBS) is added. The 15ml tubes were spun for 5 minutes at 1750xg to pellet the cells. The supernatant was discarded and 1.8 ml of RLT lysis buffer is added to the mononuclear cell pellet. The buffer and cells were pipetted up and down to ensure complete lysis of the pellet. The cell lysate was frozen and stored until it is convenient to proceed with isolation of total RNA.

Total RNA was purified from the lysed mononuclear cells using the Qiagen Rneasy Miniprep kit, as directed by the manufacturer (10/99 version) for total RNA isolation, including homogenization (Qias shredder columns) and on-column DNase treatment. The purified RNA was eluted in 50ul of water. The further use of RNA prepared by this method is described in Example 11, 24, and 23.

Some samples were prepared by a different protocol, as follows:

Two 8 ml blood samples were drawn from a peripheral vein into a tube (CPT, Becton-Dickinson order #362753) containing anticoagulant (Citrate), 25°C density gradient solution (Ficoll) and a polyester gel barrier that upon centrifugation is permeable to RBCs and granulocytes but not to mononuclear cells. The mononuclear cells and plasma remained above the barrier while the RBCs and granulocytes were

trapped below. The tube was inverted several times to mix the blood with the anticoagulant, and the tubes were subjected to centrifugation at 1750xg in a swing-out rotor at room temperature for 20 min. The tubes were removed from the centrifuge, and the clear plasma layer above the cloudy mononuclear cell layer was aspirated and discarded. The cloudy mononuclear cell layer was aspirated, with care taken to rinse all of the mononuclear cells from the surface of the gel barrier with PBS (phosphate buffered saline). Approximately 2 mls of mononuclear cell suspension was transferred to a 2ml microcentrifuge tube, and centrifuged for 3min. at 16,000 rpm in a microcentrifuge to pellet the cells. The supernatant was discarded and 1.8 ml of RLT lysis buffer (Qiagen) were added to the mononuclear cell pellet, which lysed the cells and inactivated Rnases. The cells and lysis buffer were pipetted up and down to ensure complete lysis of the pellet. Cell lysate was frozen and stored until it was convenient to proceed with isolation of total RNA.

RNA samples were isolated from 8 mL of whole blood. Yields ranged from 2 ug to 20ug total RNA for 8mL blood. A260/A280 spectrophotometric ratios were between 1.6 and 2.0, indicating purity of sample. 2ul of each sample were run on an agarose gel in the presence of ethidium bromide. No degradation of the RNA sample and no DNA contamination was visible.

Example 9: Preparation of Buffy Coat Control RNA for use in leukocyte expression profiling

Control RNA was prepared using total RNA from Buffy coats and/or total RNA from enriched mononuclear cells isolated from Buffy coats, both with and without stimulation with ionomycin and PMA. The following control RNAs were prepared:

Control 1: Buffy Coat Total RNA

Control 2: Mononuclear cell Total RNA

Control 3: Stimulated buffy coat Total RNA

Control 4: Stimulated mononuclear Total RNA

Control 5: 50% Buffy coat Total RNA / 50% Stimulated buffy coat Total

RNA

Control 6: 50% Mononuclear cell Total RNA / 50% Stimulated Mononuclear Total RNA

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Some samples were prepared using the following protocol: Buffy coats from 38 individuals were obtained from Stanford Blood Center. Each buffy coat is derived from ~350 mL whole blood from one individual. 10 ml buffy coat was removed from the bag, and placed into a 50 ml tube. 40 ml of Buffer EL (Qiagen) was added, the tube was mixed and placed on ice for 15 minutes, then cells were pelleted by centrifugation at 2000xg for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was re-suspended in 10 ml of Qiagen Buffer EL. The tube was then centrifuged at 2000xg for 10 minutes at 4°C. The cell pellet was then re-suspended in 20 ml TRIZOL (GibcoBRL) per Buffy coat sample, the mixture was shredded using a rotary homogenizer, and the lysate was then frozen at -80°C prior to proceeding to RNA isolation.

Other control RNAs were prepared from enriched mononuclear cells prepared from Buffy coats. Buffy coats from Stanford Blood Center were obtained, as described above. 10 ml buffy coat was added to a 50 ml polypropylene tube, and 10 ml of phosphate buffer saline (PBS) was added to each tube. A polysucrose (5.7 g/dL) and sodium diatrizoate (9.0 g/dL) solution at a 1.077 +/-0.0001 g/ml density solution of equal volume to diluted sample was prepared (Histopaque 1077, Sigma cat. no 1077-1). This and all subsequent steps were performed at room temperature. 15 ml of diluted buffy coat/PBS was layered on top of 15 ml of the histopaque solution in a 50 ml tube. The tube was centrifuged at 400xg for 30 minutes at room temperature. After centrifugation, the upper layer of the solution to within 0.5 cm of the opaque interface containing the mononuclear cells was discarded. The opaque interface was transferred into a clean centrifuge tube. An equal volume of PBS was added to each tube and centrifuged at 350xg for 10 minutes at room temperature. The supernatant was discarded. 5 ml of Buffer EL (Qiagen) was used to resuspend the remaining cell pellet and the tube was centrifuged at 2000xg for 10 minutes at room temperature. The supernatant was discarded. The pellet was resuspended in 20 ml of TRIZOL (GibcoBRL) for each individual buffy coat that was processed. The sample was homogenized using a rotary homogenizer and frozen at -80C until RNA was isolated.

RNA was isolated from frozen lysed Buffy coat samples as follows: frozen samples were thawed, and 4 ml of chloroform was added to each buffy coat sample. The sample was mixed by vortexing and centrifuged at 2000xg for 5 minutes. The aqueous layer was moved to new tube and then repurified by using the RNeasy Maxi

RNA clean up kit, according to the manufacturer's instruction (Qiagen, PN 75162). The yield, purity and integrity were assessed by spectrophotometer and gel electrophoresis.

Some samples were prepared by a different protocol, as follows. The further use of RNA prepared using this protocol is described in Example 11.

50 whole blood samples were randomly selected from consented blood donors at the Stanford Medical School Blood Center. Each buffy coat sample was produced from ~350 mL of an individual's donated blood. The whole blood sample was centrifuged at ~4,400 x g for 8 minutes at room temperature, resulting in three distinct layers: a top layer of plasma, a second layer of buffy coat, and a third layer of red blood cells. 25 ml of the buffy coat fraction was obtained and diluted with an equal volume of PBS (phosphate buffered saline). 30 ml of diluted buffy coat was layered onto 15 ml of sodium diatrizoate solution adjusted to a density of 1.077+/-0.001 g/ml (Histopaque 1077, Sigma) in a 50mL plastic tube. The tube was spun at 800 g for 10 minutes at room temperature. The plasma layer was removed to the 30 ml mark on the tube, and the mononuclear cell layer removed into a new tube and washed with an equal volume of PBS, and collected by centrifugation at 2000 g for 10 minutes at room temperature. The cell pellet was resuspended in 10 ml of Buffer EL (Qiagen) by vortexing and incubated on ice for 10 minutes to remove any remaining erythrocytes. The mononuclear cells were spun at 2000 g for 10 minutes at 4 degrees Celsius. The cell pellet was lysed in 25 ml of a phenol/guanidinium thiocyanate solution (TRIZOL Reagent, Invitrogen). The sample was homogenized using a PowerGene 5 rotary homogenizer (Fisher Scientific) and Omini disposable generator probes (Fisher Scientific). The Trizol lysate was frozen at -80 degrees C until the next step.

The samples were thawed out and incubated at room temperature for 5 minutes. 5 ml chloroform was added to each sample, mixed by vortexing, and incubated at room temperature for 3 minutes. The aqueous layers were transferred to new 50 ml tubes. The aqueous layer containing total RNA was further purified using the Qiagen RNeasy Maxi kit (PN 75162), per the manufacturer's protocol (October 1999). The columns were eluted twice with 1 ml Rnase-free water, with a minute incubation before each spin. Quantity and quality of RNA was assessed using standard methods. Generally, RNA was isolated from batches of 10 buffy coats at a

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time, with an average yield per buffy coat of 870 µg, and an estimated total yield of 43.5 mg total RNA with a 260/280 ratio of 1.56 and a 28S/18S ratio of 1.78.

Quality of the RNA was tested using the Agilent 2100 Bioanalyzer using RNA 6000 microfluidics chips. Analysis of the electrophorograms from the Bioanalyzer for five different batches demonstrated the reproducibility in quality between the batches.

Total RNA from all five batches were combined and mixed in a 50 ml tube, then aliquoted as follows: 2 x 10 ml aliquots in 15 ml tubes, and the rest in 100 µl aliquots in 1.5 ml microcentrifuge tubes. The aliquots gave highly reproducible results with respect to RNA purity, size and integrity. The RNA was stored at -80°C.

Test hybridization of Reference RNA

The reference RNA (hereinafter, "R50") was hybridized to a spotted cDNA array (prepared as described in Example 10). There are a total of 1152 features on the array: 384 clones printed in triplicate. The R50 targets were fluorescently labeled with Cy-5 using methods described herein. In five array hybridizations, the reference RNA detected 94% of probes on the array with a Signal to Noise ratio of greater than three. 99% of probes on the array were detected with a signal to noise ratio of greater than one. Figure 8 shows one array hybridization. The probes are ordered from high to low in signal to noise ratio, and the log of median and the log of the background were plotted for each probe.

Example 10. RNA Labeling and hybridization to a leukocyte cDNA array of candidate nucleotide sequences.

Comparison of Guanidine-Silica to Acid-Phenol RNA Purification (GSvsAP)

These data are from a set of 12 hybridizations designed to identify differences between the signal strength from two different RNA purification methods. The two RNA methods used were guanidine-silica (GS, Qiagen) and acid-phenol (AP, Trizol, Gibco BRL). Ten tubes of blood were drawn from each of four people. Two were used for the AP prep, the other eight were used for the GS prep. The protocols for the leukocyte RNA preps using the AP and GS techniques were completed as described here:

Guanidine-silica (GS) method:

For each tube, 8ml blood was drawn into a tube containing the anticoagulant Citrate, 25°C density gradient solution and a polyester gel barrier that upon centrifugation is permeable to RBCs and granulocytes but not to mononuclear cells.

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The mononuclear cells and plasma remained above the barrier while the RBCs and granulocytes were trapped below. CPT tubes from Becton-Dickinson (#362753) were used for this purpose. The tube was inverted several times to mix the blood with the anticoagulant. The tubes were immediately centrifuged @1750xg in a swinging bucket rotor at room temperature for 20 min. The tubes were removed from the centrifuge and inverted 5-10 times. This mixed the plasma with the mononuclear cells, while the RBCs and the granulocytes remained trapped beneath the gel barrier. The plasma/mononuclear cell mix was decanted into a 15ml tube and 5ml of phosphate-buffered saline (PBS) was added. The 15ml tubes are spun for 5 minutes at 1750xg to pellet the cells. The supernatant was discarded and 1.8 ml of RLT lysis buffer (guanidine isothiocyanate) was added to the mononuclear cell pellet. The buffer and cells were pipetted up and down to ensure complete lysis of the pellet. The cell lysate was then processed exactly as described in the Qiagen Rneasy Miniprep kit protocol (10/99 version) for total RNA isolation (including steps for homogenization (Qias shredder columns) and on-column DNase treatment. The purified RNA was eluted in 50ul of water.

Acid-phenol (AP) method:

For each tube, 8ml blood was drawn into a tube containing the anticoagulant Citrate, 25°C density gradient solution and a polyester gel barrier that upon centrifugation is permeable to RBCs and granulocytes but not to mononuclear cells. The mononuclear cells and plasma remained above the barrier while the RBCs and granulocytes were trapped below. CPT tubes from Becton-Dickinson (#362753) were used for this purpose. The tube was inverted several times to mix the blood with the anticoagulant. The tubes were immediately centrifuged @1750xg in a swinging bucket rotor at room temperature for 20 min. The tubes were removed from the centrifuge and inverted 5-10 times. This mixed the plasma with the mononuclear cells, while the RBCs and the granulocytes remained trapped beneath the gel barrier. The plasma/mononuclear cell mix was decanted into a 15ml tube and 5ml of phosphate-buffered saline (PBS) was added. The 15ml tubes are spun for 5 minutes @1750xg to pellet the cells. The supernatant was discarded and the cell pellet was lysed using 0.6 mL Phenol/guanidine isothiocyanate (e.g. Trizol reagent, GibcoBRL). Subsequent total RNA isolation proceeded using the manufacturers protocol.

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RNA from each person was labeled with either Cy3 or Cy5, and then hybridized in pairs to the mini-array. For instance, the first array was hybridized with GS RNA from one person (Cy3) and GS RNA from a second person (Cy5).

Techniques for labeling and hybridization for all experiments discussed here were completed as detailed above in example 10. Arrays were prepared as described in example 7.

RNA isolated from subject samples, or control Buffy coat RNA, were labeled for hybridization to a cDNA array. Total RNA (up to 100 µg) was combined with 2 µl of 100 µM solution of an Oligo (dT)12-18 (GibcoBRL) and heated to 70°C for 10 minutes and place on ice. Reaction buffer was added to the tube, to a final concentration of 1xRT buffer (GibcoBRL), 10 mM DTT (GibcoBRL), 0.1 mM unlabeled dATP, dTTP, and dGTP, and 0.025 mM unlabeled dCTP, 200 pg of CAB (*A. thaliana* photosystem I chlorophyll a/b binding protein), 200 pg of RCA (*A. thaliana* RUBISCO activase), 0.25 mM of Cy-3 or Cy-5 dCTP, and 400 U Superscript II RT (GibcoBRL).

The volumes of each component of the labeling reaction were as follows: 20 µl of 5xRT buffer; 10 µl of 100 mM DTT; 1 µl of 10 mM dNTPs without dCTP; 0.5 µl of 5 mM CTP; 13 µl of H₂O; 0.02 µl of 10 ng/µl CAB and RCA; 1 µl of 40 Units/µl RNaseOUT Recombinant Ribonuclease Inhibitor (GibcoBRL); 2.5 µl of 1.0 mM Cy-3 or Cy-5 dCTP; and 2.0 µl of 200 Units/µl of Superscript II RT. The sample was vortexed and centrifuged. The sample was incubated at 4°C for 1 hour for first strand cDNA synthesis, then heated at 70°C for 10 minutes to quench enzymatic activity. 1 µl of 10 mg/ml of Rnase A was added to degrade the RNA strand, and the sample was incubated at 37°C for 30 minutes.

Next, the Cy-3 and Cy-5 cDNA samples were combined into one tube. Unincorporated nucleotides were removed using QIAquick RCR purification protocol (Qiagen), as directed by the manufacturer. The sample was evaporated to dryness and resuspended in 5 µl of water. The sample was mixed with hybridization buffer containing 5xSSC, 0.2% SDS, 2 mg/ml Cot-1 DNA (GibcoBRL), 1 mg/ml yeast tRNA (GibcoBRL), and 1.6 ng/µl poly dA40-60 (Pharmacia). This mixture was placed on the microarray surface and a glass cover slip was placed on the array (Corning). The microarray glass slide was placed into a hybridization chamber (ArrayIt). The chamber was then submerged in a water bath overnight at 62° C. The

microarray was removed from the cassette and the cover slip was removed by repeatedly submerging it to a wash buffer containing 1xSSC, and 0.1% SDS. The microarray slide was washed in 1xSSC/0.1% SDS for 5 minutes. The slide was then washed in 0.1xSSC/0.1% SDS for 5 minutes. The slide was finally washed in 0.1xSSC for 2 minutes. The slide was spun at 1000 rpm for 2 minutes to dry out the slide, then scanned on a microarray scanner (Axon Instruments, Union City, CA.).

Six hybridizations with 20 µg of RNA were performed for each type of RNA preparation (GS or AP). Since both the Cy3 and the Cy5 labeled RNA are from test preparations, there are six data points for each GS prepped, Cy3-labeled RNA and six for each GS-prepped, Cy5-labeled RNA. The mini array hybridizations were scanned on an Axon Instruments scanner using GenPix 3.0 software. The data presented were derived as follows. First, all features flagged as "not found" by the software were removed from the dataset for individual hybridizations. These features are usually due to high local background or other processing artifacts. Second, the median fluorescence intensity minus the background fluorescence intensity was used to calculate the mean background subtracted signal for each dye for each hybridization. In Figure 4, the mean of these means across all six hybridizations is graphed (n=6 for each column). The error bars are the SEM. This experiment shows that the average signal from AP prepared RNA is 47% of the average signal from GS prepared RNA for both Cy3 and Cy5.

Generation of expression data for leukocyte genes from peripheral leukocyte samples

Six hybridizations were performed with RNA purified from human blood leukocytes using the protocols given above. Four of the six were prepared using the GS method and 2 were prepared using the AP method. Each preparation of leukocyte RNA was labeled with Cy3 and 10 µg hybridized to the mini-array. A control RNA was batch labeled with Cy5 and 10 µg hybridized to each mini-array together with the Cy3-labeled experimental RNA.

The control RNA used for these experiments was Control 1: Buffy Coat RNA, as described above. The protocol for the preparation of that RNA is reproduced here:

Buffy Coat RNA Isolation:

Buffy coats were obtained from Stanford Blood Center (in total 38 individual buffy coats were used. Each buffy coat is derived from ~350 mL whole blood from

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one individual. 10 ml buffy coat was taken and placed into a 50 ml tube and 40 ml of a hypochlorous acid (HOCl) solution (Buffer EL from Qiagen) was added. The tube was mixed and placed on ice for 15 minutes. The tube was then centrifuged at 2000xg for 10 minutes at 4°C. The supernatant was decanted and the cell pellet was re-suspended in 10 ml of hypochlorous acid solution (Qiagen Buffer EL). The tube was then centrifuged at 2000xg for 10 minutes at 4°C. The cell pellet was then re-suspended in 20 ml phenol/guanidine thiocyanate solution (TRIZOL from GibcoBRL) for each individual buffy coat that was processed. The mixture was then shredded using a rotary homogenizer. The lysate was then frozen at -80°C prior to proceeding to RNA isolation.

The arrays were then scanned and analyzed on an Axon Instruments scanner using GenePix 3.0 software. The data presented were derived as follows. First, all features flagged as "not found" by the software were removed from the dataset for individual hybridizations. Second, control features were used to normalize the data for labeling and hybridization variability within the experiment. The control features are cDNA for genes from the plant, *Arabidopsis thaliana*, that were included when spotting the mini-array. Equal amounts of RNA complementary to two of these cDNAs were added to each of the samples before they were labeled. A third was pre-labeled and equal amounts were added to each hybridization solution before hybridization. Using the signal from these genes, we derived a normalization constant (L_j) according to the following formula:

$$L_j = \frac{\frac{\sum_{i=1}^N BGSS_{j,i}}{N}}{\frac{\sum_{j=1}^K \frac{\sum_{i=1}^N BGSS_{j,i}}{N}}{K}}$$

where $BGSS_i$ is the signal for a specific feature as identified in the GenePix software as the median background subtracted signal for that feature, N is the number of *A. thaliana* control features, K is the number of hybridizations, and L is the normalization constant for each individual hybridization.

Using the formula above, the mean over all control features of a particular hybridization and dye (eg Cy3) was calculated. Then these control feature means for all Cy3 hybridizations were averaged. The control feature mean in one hybridization divided by the average of all hybridizations gives a normalization constant for that particular Cy3 hybridization.

The same normalization steps were performed for Cy3 and Cy5 values, both fluorescence and background. Once normalized, the background Cy3 fluorescence was subtracted from the Cy3 fluorescence for each feature. Values less than 100 were eliminated from further calculations since low values caused spurious results.

Figure 5 shows the average background subtracted signal for each of nine leukocyte-specific genes on the mini array. This average is for 3-6 of the above-described hybridizations for each gene. The error bars are the SEM. Figure 3: The ratio of Cy3 to Cy5 signal is shown for a number of genes. This ratio corrects for variability among hybridizations and allows comparison between experiments done at different times. The ratio is calculated as the Cy3 background subtracted signal divided by the Cy5 background subtracted signal. Each bar is the average for 3-6 hybridizations. The error bars are SEM.

Together, these results show that we can measure expression levels for genes that are expressed specifically in sub-populations of leukocytes. These expression measurements were made with only 10 µg of leukocyte total RNA that was labeled directly by reverse transcription. The signal strength can be increased by improved labeling techniques that amplify either the starting RNA or the signal fluorescence. In addition, scanning techniques with higher sensitivity can be used.

Genes in Figures 5 and 6:

Gene Name/Description	GenBank Accession Number	Gene Name Abbreviation
T cell-specific tyrosine kinase Mrna	L10717	TKTCS
Interleukin 1 alpha (IL 1) mRNA, complete cds	NM_000575	IL1A
T-cell surface antigen CD2 (T11) mRNA, complete cds	M14362	CD2
Interleukin-13 (IL-13) precursor gene, complete cds	U31120	IL-13
Thymocyte antigen CD1a mRNA, complete cds	M28825	CD1a

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CD6 mRNA for T cell glycoprotein CDS	NM_006725	CD6
MHC class II HLA-DQA1 mRNA, complete cds	U77589	HLA-DQA1
Granulocyte colony-stimulating factor	M28170	CD19
Homo sapiens CD69 antigen	NM_001781	CD69

Example 11: Identification of diagnostic gene sets useful in diagnosis and treatment of Cardiac allograft rejection

An observational study was conducted in which a prospective cohort of cardiac transplant recipients were analyzed for associations between clinical events or rejection grades and expression of a leukocyte candidate nucleotide sequence library. Patients were identified at 4 cardiac transplantation centers while on the transplant waiting list or during their routing post-transplant care. All adult cardiac transplant recipients (new or re-transplants) who received an organ at the study center during the study period or within 3 months of the start of the study period were eligible. The first year after transplantation is the time when most acute rejection occurs and it is thus important to study patients during this period. Patients provided informed consent prior to study procedures.

Peripheral blood leukocyte samples were obtained from all patients at the following time points: prior to transplant surgery (when able), the same day as routinely scheduled screening biopsies, upon evaluation for suspected acute rejection (urgent biopsies), on hospitalization for an acute complication of transplantation or immunosuppression, and when Cytomegalovirus (CMV) infection was suspected or confirmed. Samples were obtained through a standard peripheral vein blood draw or through a catheter placed for patient care (for example, a central venous catheter placed for endocardial biopsy). When blood was drawn from a intravenous line, care was taken to avoid obtaining heparin with the sample as it can interfere with downstream reactions involving the RNA. Mononuclear cells were prepared from whole blood samples as described in Example 8. Samples were processed within 2 hours of the blood draw and DNA and serum were saved in addition to RNA. Samples were stored at -70°C or on dry ice and sent to the site of RNA preparation in a sealed container with ample dry ice. RNA was isolated from subject samples as

described in Example 8 and hybridized to a candidate library of differentially expressed leukocyte nucleotide sequences, as further described in Examples 20-22. Methods used for amplification, labeling, hybridization and scanning are described in example 23. Analysis of human transplant patient mononuclear cell RNA hybridized to a microarray is shown in Example 24.

From each patient, clinical information was obtained at the following time points: prior to transplant surgery (when available), the same day as routinely scheduled screening biopsies, upon evaluation for suspected acute rejection (e.g., urgent biopsies), on hospitalization for an acute complication of transplantation or immunosuppression, and when Cytomegalovirus (CMV) infection was suspected or confirmed. Data was collected directly from the patient, from the patient's medical record, from diagnostic test reports or from computerized hospital databases. It was important to collect all information pertaining to the study clinical correlates (diagnoses and patient events and states to which expression data is correlated) and confounding variables (diagnoses and patient events and states that may result in altered leukocyte gene expression. Examples of clinical data collected are: patient sex, date of birth, date of transplant, race, requirement for prospective cross match, occurrence of pre-transplant diagnoses and complications, indication for transplantation, severity and type of heart disease, history of left ventricular assist devices, all known medical diagnoses, blood type, HLA type, viral serologies (including CMV, Hepatitis B and C, HIV and others), serum chemistries, white and red blood cell counts and differentials, CMV infections (clinical manifestations and methods of diagnosis), occurrence of new cancer, hemodynamic parameters measured by catheterization of the right or left heart (measures of graft function), results of echocardiography, results of coronary angiograms, results of intravascular ultrasound studies (diagnosis of transplant vasculopathy), medications, changes in medications, treatments for rejection, and medication levels. Information was also collected regarding the organ donor, including demographics, blood type, HLA type, results of screening cultures, results of viral serologies, primary cause of brain death, the need for inotropic support, and the organ cold ischemia time.

Of great importance was the collection of the results of endocardial biopsy for each of the patients at each visit. Biopsy results were all interpreted and recorded using the international society for heart and lung transplantation (ISHLT) criteria, described below. Biopsy pathological grades were determined by experienced

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pathologists at each center. It is desirable to have a single centralized pathologist determine the grades when an analysis is done using samples from multiple medical centers.

ISHLT Criteria

Grade	Finding	Rejection Severity
0	No lymphocytic infiltrates	None
1A	Focal (perivascular or interstitial lymphocytic infiltrates without necrosis)	Borderline mild
1B	Diffuse but sparse lymphocytic infiltrates without necrosis	Mild
2	One focus only with aggressive lymphocytic infiltrate and/or myocyte damage	Mild, focal moderate
3A	Multifocal aggressive lymphocytic infiltrates and/or myocardial damage	Moderate
3B	Diffuse inflammatory lymphocytic infiltrates with necrosis	Borderline Severe
4	Diffuse aggressive polymorphous lymphocytic infiltrates with edema hemorrhage and vasculitis, with necrosis	Severe

Clinical data was entered and stored in a database. The database was queried to identify all patients and patient visits that meet desired criteria (for example, patients with > grade II biopsy results, no CMV infection and time since transplant < 12 weeks).

The collected clinical data (disease criteria) is used to define patient or sample groups for correlation of expression data. Patient groups are identified for comparison, for example, a patient group that possesses a useful or interesting clinical distinction, versus a patient group that does not possess the distinction. Examples of useful and interesting patient distinctions that can be made on the basis of collected clinical data are listed here (and further described in Table 2):

1. Rejection episode of at least moderate histologic grade, which results in treatment of the patient with additional corticosteroids, anti-T cell antibodies, or total lymphoid irradiation.

2. Rejection with histologic grade 2 or higher.
3. Rejection with histologic grade <2 .
4. The absence of histologic rejection and normal or unchanged allograft function (based on hemodynamic measurements from catheterization or on echocardiographic data).
5. The presence of severe allograft dysfunction or worsening allograft dysfunction during the study period (based on hemodynamic measurements from catheterization or on echocardiographic data).
6. Documented CMV infection by culture, histology, or PCR, and at least one clinical sign or symptom of infection.
7. Specific graft biopsy rejection grades
8. Rejection of mild to moderate histologic severity prompting augmentation of the patient's chronic immunosuppressive regimen.
9. Rejection of mild to moderate severity with allograft dysfunction prompting plasmaphoresis or a diagnosis of "humoral" rejection
10. Infections other than CMV, esp. Epstein Barr virus (EBV)
11. Lymphoproliferative disorder (also called, post-transplant lymphoma)
12. Transplant vasculopathy diagnosed by increased intimal thickness on intravascular ultrasound (IVUS), angiography, or acute myocardial infarction.
13. Graft Failure or Retransplantation
14. All cause mortality

Expression profiles of subject samples are examined to discover sets of nucleotide sequences with differential expression between patient groups, for example, by methods describes above and below.

Non-limiting examples of patient leukocyte samples to obtain for discovery of various diagnostic nucleotide sets are as follows:

- a. Leukocyte set to avoid biopsy or select for biopsy:
Samples : Grade 0 vs. Grades 1-4
- b. Leukocyte set to monitor therapeutic response:
Examine successful vs. unsuccessful drug treatment.

Samples:

Successful: Time 1: rejection, Time 2: drug therapy Time 3: no rejection

Unsuccessful: Time 1: rejection, Time 2: drug therapy; Time 3: rejection

- c. Leukocyte set to predict subsequent acute rejection.
Biopsy may show no rejection, but the patient may develop rejection shortly thereafter. Look at profiles of patients who subsequently do and do not develop rejection.

Samples:

Group 1 (Subsequent rejection): Time 1: Grade 0; Time 2: Grade >0

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Group 2 (No subsequent rejection): Time 1: Grade 0, ; Time 2: Grade 0

Focal rejection may be missed by biopsy. When this occurs the patient may have a Grade 0, but actually has rejection. These patients may go on to have damage to the graft etc.

Samples:

Non-rejectors: no rejection over some period of time

Rejectors: an episode of rejection over same period

- d. Leukocyte set to diagnose subsequent or current graft failure:

Samples:

Echocardiographic or catheterization data to define worsening function over time and correlate to profiles.

- e. Leukocyte set to diagnose impending active CMV:

Samples:

Look at patients who are CMV IgG positive. Compare patients with subsequent (to a sample) clinical CMV infection verses no subsequent clinical CMV infection.

- f. Leukocyte set to diagnose current active CMV:

Samples:

Analyze patients who are CMV IgG positive. Compare patients with active current clinical CMV infection vs. no active current CMV infection.

Upon identification of a nucleotide sequence or set of nucleotide sequences that distinguish patient groups with a high degree of accuracy, that nucleotide sequence or set of nucleotide sequences is validated, and implemented as a diagnostic test. The use of the test depends on the patient groups that are used to discover the nucleotide set. For example, if a set of nucleotide sequences is discovered that have collective expression behavior that reliably distinguishes patients with no histological rejection or graft dysfunction from all others, a diagnostic is developed that is used to screen patients for the need for biopsy. Patients identified as having no rejection do not need biopsy, while others are subjected to a biopsy to further define the extent of disease. In another example, a diagnostic nucleotide set that determines continuing graft rejection associated with myocyte necrosis ($>$ grade I) is used to determine that a patient is not receiving adequate treatment under the current treatment regimen. After increased or altered immunosuppressive therapy, diagnostic profiling is conducted to

determine whether continuing graft rejection is progressing. In yet another example, a diagnostic nucleotide set(s) that determine a patient's rejection status and diagnose cytomegalovirus infection is used to balance immunosuppressive and anti-viral therapy.

Example 12: Identification of diagnostic nucleotide sets for kidney and liver allograft rejection

Diagnostic tests for rejection are identified using patient leukocyte expression profiles to identify a molecular signature correlated with rejection of a transplanted kidney or liver. Blood, or other leukocyte source, samples are obtained from patients undergoing kidney or liver biopsy following liver or kidney transplantation, respectively. Such results reveal the histological grade, i.e., the state and severity of allograft rejection. Expression profiles are obtained from the samples as described above, and the expression profile is correlated with biopsy results. In the case of kidney rejection, clinical data is collected corresponding to urine output, level of creatine clearance, and level of serum creatine (and other markers of renal function). Clinical data collected for monitoring liver transplant rejection includes, biochemical characterization of serum markers of liver damage and function such as SGOT, SGPT, Alkaline phosphatase, GGT, Bilirubin, Albumin and Prothrombin time.

Leukocyte nucleotide sequence expression profiles are collected and correlated with important clinical states and outcomes in renal or hepatic transplantation. Examples of useful clinical correlates are given here:

1. Rejection episode of at least moderate histologic grade, which results in treatment of the patient with additional corticosteroids, anti-T cell antibodies, or total lymphoid irradiation.
2. The absence of histologic rejection and normal or unchanged allograft function (based on tests of renal or liver function listed above).
3. The presence of severe allograft dysfunction or worsening allograft dysfunction during the study period (based on tests of renal and hepatic function listed above).
4. Documented CMV infection by culture, histology, or PCR, and at least one clinical sign or symptom of infection.
5. Specific graft biopsy rejection grades
6. Rejection of mild to moderate histologic severity prompting augmentation of the patient's chronic immunosuppressive regimen
7. Infections other than CMV, esp. Epstein Barr virus (EBV)
8. Lymphoproliferative disorder (also called, post-transplant lymphoma)
9. Graft Failure or Retransplantation
10. Need for hemodialysis or other renal replacement therapy for renal transplant patients.

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11. Hepatic encephalopathy for liver transplant recipients.
12. All cause mortality

Subsets of the candidate library (or of a previously identified diagnostic nucleotide set), are identified, according to the above procedures, that have predictive and/or diagnostic value for kidney or liver allograft rejection.

Example 13: Identification of diagnostic nucleotide sequences sets for use in the diagnosis, prognosis, risk stratification, and treatment of Atherosclerosis, Stable Angina Pectoris, and acute coronary syndrome.

Prediction of complications of atherosclerosis: angina pectoris.

Over 50 million in the US have atherosclerotic coronary artery disease (CAD). Almost all adults have some atherosclerosis. The most important question is who will develop complications of atherosclerosis. Patients with angiographically-confirmed atherosclerosis are enrolled in a study, and followed over time. Leukocyte expression profiles are taken at the beginning of the study, and routinely thereafter. Some patients develop angina and others do not. Expression profiles are correlated with development of angina, and subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive and/or diagnostic value for angina pectoris.

Alternatively, patients are followed by serial angiography. Profiles are collected at the first angiography, and at a repeat angiography at some future time (for example, after 1 year). Expression profiles are correlated with progression of disease, measured, for example, by decrease in vessel lumen diameter. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive and/or diagnostic value for progression of atherosclerosis.

Prediction and/or diagnosis of acute coronary syndrome

The main cause of death due to coronary atherosclerosis is the occurrence of acute coronary syndromes: myocardial infarction and unstable angina. Patients with at a very high risk of acute coronary syndrome (e.g., patients with a history of acute coronary syndrome, patients with atherosclerosis, patients with multiple traditional risk factors, clotting disorders or lupus) are enrolled in a prospective study. Leukocyte expression profiles are taken at the beginning of the study period and patients are monitored for the occurrence of unstable angina and/or myocardial

infarction. Standard criteria for the occurrence of an event are used (serum enzyme elevation, EKG, nuclear imaging or other), and the occurrence of these events can be collected from the patient, the patient's physician, the medical record or medical database. Expression profiles (taken at the beginning of the study) are correlated with the occurrence of an acute event. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for occurrence of an acute event.

In addition, expression profiles (taken at the time that an acute event occurs) are correlated with the occurrence of an acute event. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have diagnostic value for occurrence of an acute event.

Risk stratification: occurrence of coronary artery disease

The established and classic risks for the occurrence of coronary artery disease and complications of that disease are: cigarette smoking, diabetes, hypertension, hyperlipidemia and a family history of early atherosclerosis. Obesity, sedentary lifestyle, syndrome X, cocaine use, chronic hemodialysis and renal disease, radiation exposure, endothelial dysfunction, elevated plasma homocysteine, elevated plasma lipoprotein a, and elevated CRP. Infection with CMV and chlamydia infection are less well established, controversial or putative risk factors for the disease. These risk factors can be assessed or measured in a population.

Leukocyte expression profiles are measured in a population possessing risk factors for the occurrence of coronary artery disease. Expression profiles are correlated with the presence of one or more risk factors (that may correlate with future development of disease and complications). Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for the development of coronary artery disease.

Additional examples of useful correlation groups in cardiology include:

1. Samples from patients with a high risk factor burden (e.g., smoking, diabetes, high cholesterol, hypertension, family history) versus samples from those same patients at different times with fewer risks, or versus samples from different patients with fewer or different risks.

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2. Samples from patients during an episode of unstable angina or myocardial infarction versus paired samples from those same patients before the episode or after recovery, or from different patients without these diagnoses.

3. Samples from patients (with or without documented atherosclerosis) who subsequently develop clinical manifestations of atherosclerosis such as stable angina, unstable angina, myocardial infarction, or stroke, versus samples from patients (with or without atherosclerosis) who do not develop these manifestations over the same time period.

4. Samples from patients who subsequently respond to a given medication or treatment regimen versus samples from those same or different patients who subsequently do not respond to a given medication or treatment regimen.

Example 14: Identification of diagnostic nucleotide sets for use in diagnosing and treating Restenosis

Restenosis is the re-narrowing of a coronary artery after an angioplasty. Patients are identified who are about to, or have recently undergone angioplasty. Leukocyte expression profiles are measured before the angioplasty, and at 1 day and 1-2 weeks after angioplasty or stent placement. Patients have a follow-up angiogram at 3 months and/or are followed for the occurrence of clinical restenosis, e.g., chest pain due to re-narrowing of the artery, that is confirmed by angiography. Expression profiles are compared between patients with and without restenosis, and candidate nucleotide profiles are correlated with the occurrence of restenosis. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for the development of restenosis.

Example 15: Identification of diagnostic nucleotide sets for use in monitoring treatment and/or progression of Congestive Heart Failure

CHF affects greater than 5 million individuals in the US and the prevalence of this disorder is growing as the population ages. The disease is chronic and debilitating. Medical expenditures are huge due to the costs of drug treatments, echocardiograms and other tests, frequent hospitalization and cardiac transplantation. The primary causes of CHF are coronary artery disease, hypertension and idiopathic

cardiomyopathy. Congestive heart failure is the number one indication for heart transplantation.

There is ample recent evidence that congestive heart failure is associated with systemic inflammation. A leukocyte test with the ability to determine the rate of progression and the adequacy of therapy is of great interest. Patients with severe CHF are identified, e.g. in a CHF clinic, an inpatient service, or a CHF study or registry (such as the cardiac transplant waiting list/registry). Expression profiles are taken at the beginning of the study and patients are followed over time, for example, over the course of one year, with serial assessments performed at least every three months. Further profiles are taken at clinically relevant end-points, for example: hospitalization for CHF, death, pulmonary edema, worsening of Ejection Fraction or increased cardiac chamber dimensions determined by echocardiography or another imaging test, and/or exercise testing of hemodynamic measurements. Clinical data is collected from patients if available, including:

Serial C-Reactive Protein (CRP), other serum markers, echocardiography (e.g., ejection fraction or another echocardiographic measure of cardiac function), nuclear imaging, NYHA functional classes, hospitalizations for CHF, quality of life measures, renal function, transplant listing, pulmonary edema, left ventricular assist device use, medication use and changes.

Expression profiles correlating with progression of CHF are identified. Expression profiles predicting disease progression, monitoring disease progression and response to treatment, and predicting response to a particular treatment(s) or class of treatment(s) are identified. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for the progression of CHF. Such diagnostic nucleotide sets are also useful for monitoring response to treatment for CHF.

Example 16: Identification of diagnostic nucleotide sets for use in monitoring treatment and/or progression of Rheumatoid arthritis

Rheumatoid arthritis (hereinafter, "RA") is a chronic and debilitating inflammatory arthritis. The diagnosis of RA is made by clinical criteria and radiographs. A new class of medication, TNF blockers, are effective, but the drugs are expensive, have side effects and not all patients respond to treatment. In addition, relief of disease symptoms does not always correlate with inhibition of joint

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destruction. For these reasons, an alternative mechanism for the titration of therapy is needed.

An observational study was conducted in which a cohort of patients meeting American College of Rheumatology (hereinafter "ARC") criteria for the diagnosis of RA was identified. Arnett et al. (1988) Arthritis Rheum 31:315-24. Patients gave informed consent and a peripheral blood mononuclear cell RNA sample was obtained by the methods as described herein. When available, RNA samples were also obtained from surgical specimens of bone or synovium from effected joints, and synovial fluid .

From each patient, the following clinical information was obtained if available:

Demographic information; information relating to the ACR criteria for RA; presence or absence of additional diagnoses of inflammatory and non-inflammatory conditions; data from laboratory test, including complete blood counts with differentials, CRP, ESR, ANA, Serum IL6, Soluble CD40 ligand, LDL, HDL, Anti-DNA antibodies, rheumatoid factor, C3, C4, serum creatinine and any medication levels; data from surgical procedures such as gross operative findings and pathological evaluation of resected tissues and biopsies; information on pharmacological therapy and treatment changes; clinical diagnoses of disease "flare"; hospitalizations; quantitative joint exams; results from health assessment questionnaires (HAQs); other clinical measures of patient symptoms and disability; physical examination results and radiographic data assessing joint involvement, synovial thickening, bone loss and erosion and joint space narrowing and deformity.

From these data, measures of improvement in RA are derived as exemplified by the ACR 20% and 50% response/improvement rates (Felson et al. 1996). Measures of disease activity over some period of time is derived from these data as are measures of disease progression. Serial radiography of effected joints is used for objective determination of progression (e.g., joint space narrowing, peri-articular osteoporosis, synovial thickening). Disease activity is determined from the clinical scores, medical history, physical exam, lab studies, surgical and pathological findings. The collected clinical data (disease criteria) is used to define patient or sample groups for correlation of expression data. Patient groups are identified for comparison; for example, a patient group that possesses a useful or interesting clinical distinction, verses a patient group that does not possess the distinction. Examples of useful and

interesting patient distinctions that can be made on the basis of collected clinical data are listed here:

1. Samples from patients during a clinically diagnosed RA flare versus samples from these same or different patients while they are asymptomatic.
2. Samples from patients who subsequently have high measures of disease activity versus samples from those same or different patients who have low subsequent disease activity.
3. Samples from patients who subsequently have high measures of disease progression versus samples from those same or different patients who have low subsequent disease progression.
4. Samples from patients who subsequently respond to a given medication or treatment regimen versus samples from those same or different patients who subsequently do not respond to a given medication or treatment regimen (for example, TNF pathway blocking medications).
5. Samples from patients with a diagnosis of osteoarthritis versus patients with rheumatoid arthritis.
6. Samples from patients with tissue biopsy results showing a high degree of inflammation versus samples from patients with lesser degrees of histological evidence of inflammation on biopsy.

Expression profiles correlating with progression of RA are identified. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for the progression of RA.

Diagnostic nucleotide set(s) are identified which predict respond to TNF blockade. Patients are profiled before and during treatment with these medications. Patients are followed for relief of symptoms, side effects and progression of joint destruction, e.g., as measured by hand radiographs. Expression profiles correlating with response to TNF blockade are identified. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures that have predictive value for response to TNF blockade.

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Example 17: Identification of diagnostic nucleotide sets for diagnosis of Systemic Lupus Erythematosus

SLE is a chronic, systemic inflammatory disease characterized by dysregulation of the immune system. Clinical manifestations affect every organ system and include skin rash, renal dysfunction, CNS disorders, arthralgias and hematologic abnormalities. SLE clinical manifestations tend to both recur intermittently (or “flare”) and progress over time, leading to permanent end-organ damage.

An observational study was conducted in which a cohort of patients meeting American College of Rheumatology (hereinafter “ACR”) criteria for the diagnosis of SLE were identified. See Tan et al. (1982) Arthritis Rheum 25:1271-7. Patients gave informed consent and a peripheral blood mononuclear cell RNA sample was obtained by the methods as described herein.

From each patient, the following clinical information was obtained if available:

Demographic information, ACR criteria for SLE, additional diagnoses of inflammatory and non-inflammatory conditions, data from laboratory testing including complete blood counts with differentials, CRP, ESR, ANA, Serum IL6, Soluble CD40 ligand, LDL, HDL, Anti-DNA antibodies, rheumatoid factor, C3, C4, serum creatinine (and other measures of renal dysfunction) and any medication levels, data from surgical procedures such as gross operative findings and pathological evaluation of resected tissues and biopsies (e.g., renal, CNS), information on pharmacological therapy and treatment changes, clinical diagnoses of disease “flare”, hospitalizations, quantitative joint exams, results from health assessment questionnaires (HAQs), SLEDAIs (a clinical score for SLE activity that assess many clinical variables), other clinical measures of patient symptoms and disability, physical examination results and carotid ultrasonography.

The collected clinical data (disease criteria) is used to define patient or sample groups for correlation of expression data. Patient groups are identified for comparison, for example, a patient group that possesses a useful or interesting clinical distinction, verses a patient group that does not possess the distinction. Measures of disease activity in SLE are derived from the clinical data described above to divide patients (and patient samples) into groups with higher and lower disease activity over some period of time or at any one point in time. Such data are SLEDAI scores and

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other clinical scores, levels of inflammatory markers or complement, number of hospitalizations, medication use and changes, biopsy results and data measuring progression of end-organ damage or end-organ damage, including progressive renal failure, carotid atherosclerosis, and CNS dysfunction. Further examples of useful and interesting patient distinctions that can be made on the basis of collected clinical data are listed here:

Samples from patients during a clinically diagnosed SLE flare versus samples from these same or different patients while they are asymptomatic or while they have a documented infection.

1. Samples from patients who subsequently have high measures of disease activity versus samples from those same or different patients who have low subsequent disease activity.

2. Samples from patients who subsequently have high measures of disease progression versus samples from those same or different patients who have low subsequent disease progression.

3. Samples from patients who subsequently respond to a given medication or treatment regimen versus samples from those same or different patients who subsequently do not respond to a given medication or treatment regimen.

4. Samples from patients with premature carotid atherosclerosis on ultrasonography versus patients with SLE without premature atherosclerosis.

Expression profiles correlating with progression of SLE are identified, including expression profiles corresponding to end-organ damage and progression of end-organ damage. Expression profiles are identified predicting disease progression or disease "flare", response to treatment or likelihood of response to treatment, predict likelihood of "low" or "high" disease measures (optionally described using the SLEDAI score), and presence or likelihood of developing premature carotid atherosclerosis. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for the progression of SLE.

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Example 18: Identification of a diagnostic nucleotide set for diagnosis of cytomegalovirus

Cytomegalovirus is a very important cause of disease in immunosuppressed patients, for example, transplant patients, cancer patients, and AIDS patients. The virus can cause inflammation and disease in almost any tissue (particularly the colon, lung, bone marrow and retina). It is increasingly important to identify patients with current or impending clinical CMV disease, particularly when immunosuppressive drugs are to be used in a patient, e.g. for preventing transplant rejection.

Leukocytes are profiled in patients with active CMV, impending CMV, or no CMV. Expression profiles correlating with diagnosis of active or impending CMV are identified. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for the diagnosis of active or impending CMV. Diagnostic nucleotide set(s) identified with predictive value for the diagnosis of active or impending CMV may be combined, or used in conjunction with, cardiac, liver and/or kidney allograft-related diagnostic gene set(s) (described in Examples 11 and 12).

In addition, or alternatively, CMV nucleotide sequences are obtained, and a diagnostic nucleotide set is designed using CMV nucleotide sequence. The entire sequence of the organism is known and all CMV nucleotide sequences can be isolated and added to the library using the sequence information and the approach described below. Known expressed genes are preferred. Alternatively, nucleotide sequences are selected to represent groups of CMV genes that are coordinately expressed (immediate early genes, early genes, and late genes) (Spector et al. 1990, Stamminger et al. 1990).

CMV nucleotide sequences were isolated as follows: Primers were designed to amplify known expressed CMV genes, based on the publically available sequence of CMV strain AD 169 (Genbank LOCUS: HEHCMVCG 229354 bp;

DEFINITION Human cytomegalovirus strain AD169 complete genome;

ACCESSION X17403; VERSION X17403.1 GI:59591). The following primer were used to PCR amplify nucleotide sequences from 175 ng of AD 169 viral genomic DNA (Advance Biotechnologies Incorporated) as a template:

CMV GENE	PRIMER SEQUENCES	SEQ. ID. NO:
UL21 5'	atgtggcgcgtctgaaaaac	8771

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US3 5'	atgaagcgggtgttggtctc	8815
US3 3'	ttaaataaatcgagacgggog	8816
US6 5'	atggatctcttgattgtctcg	8817
US6 3'	tcaggagccacaacgtogaatc	8818
US11 5'	cgcaaaacgctactggctoc	8819
US11 3'	tcaccactggtcgaaaacatc	8820
US18 5'	taaggctggtcgtcatcgt	8821
US18 3'	ttacaacaagctgaggagactc	8822
US27 5'	atgaccacctctacaataatcaaac	8823
US27 3'	gtagaacaagcgttgagtccc	8824
US28 5'	cgttgcggtgtctcagtcg	8825
US28 3'	tcatgtctgtgtaccaggata	8826

The PCR reaction conditions were 10 mM Tris pH 8.3, 3.5 mM MgCl₂, 25 mM KCl, 200 uM dNTP's, 0.2 uM primers, and 5 Units of Taq Gold. The cycle parameters were as follows:

1. 95°C for 30 sec
2. 95°C for 15 sec
3. 56°C for 30 sec
4. 72°C for 2 min
5. go to step 2, 29 times
6. 72°C for 2 min
7. 4°C forever

PCR products were gel purified, and DNA was extracted from the agarose using the QiaexII gel purification kit (Qiagen). PCR product was ligated into the T/A cloning vector p-GEM-T-Easy (Promega) using 3 ul of gel purified PCR product and following the Promega protocol. The products of the ligation reaction were transformed and plated as described in the p-GEM protocol. White colonies were picked and grow culture in LB-AMP medium. Plasmid was prepared from these cultures using Qiagen Miniprep kit (Qiagen). Restriction enzyme digested plasmid (Not I and EcoRI) was examined after agarose gel electrophoresis to assess insert size. When the insert was the predicted size, the plasmid was sequenced by well-known techniques to confirm the identity of the CMV gene. Using forward and reverse primers that are complimentary to sequences flanking the insert cloning site (M13F and M13R), the isolated CMV gene was amplified and purified as described above.

Amplified cDNAs were used to create a microarray as described above. In addition, 50mer oligonucleotides corresponding the CMV genes listed above were designed, synthesized and placed on a microarray using methods described elsewhere in the specification.

Alternatively, oligonucleotide sequences are designed and synthesized for oligonucleotide array expression analysis from CMV genes as described in examples 20-22.

Diagnostic nucleotide set(s) for expression of CMV genes is used in combination with diagnostic leukocyte nucleotide sets for diagnosis of other conditions, e.g. organ allograft rejection.

Example 19: Identification of diagnostic nucleotide sets for monitoring response to Statins

HMG-CoA reductase inhibitors, called "Statins," are very effective in preventing complications of coronary artery disease in either patients with coronary disease and high cholesterol (secondary prevention) or patients without known coronary disease and with high cholesterol (primary prevention). Examples of Statins are (generic names given) pravastatin, atorvastatin, and simvastatin. Monitoring response to Statin therapy is of interest. Patients are identified who are on or are about to start Statin therapy. Leukocytes are profiled in patients before and after initiation of therapy, or in patients already being treated with Statins. Data is collected corresponding to cholesterol level, markers of inflammation (e.g., C-Reactive Protein and the Erythrocyte Sedimentation Rate), measures of endothelial function (e.g., improved forearm resistance or coronary flow reserve) and clinical endpoints (new stable angina, unstable angina, myocardial infarction, ventricular arrhythmia, claudication). Patient groups can be defined based on their response to Statin therapy (cholesterol, clinical endpoints, endothelial function). Expression profiles correlating with response to Statin treatment are identified. Subsets of the candidate library (or a previously identified diagnostic nucleotide set) are identified, according to the above procedures, that have predictive value for the response to Statins. Members of candidate nucleotide sets with expression that is altered by Statins are disease target nucleotides sequences.

Example 20—Probe Selection for a 24,000 Feature Array

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This Example describes the compilation of almost 8,000 unique genes and ESTs using sequences identified from the sources described below. The sequences of these genes and ESTs were used to design probes, as described in the following Example.

Tables 3A, 3B and 3C list the sequences identified in the subtracted leukocyte expression libraries. All sequences that were identified as corresponding to a known RNA transcript were represented at least once, and all unidentified sequences were represented twice – once by the sequence on file and again by the complementary sequence – to ensure that the sense (or coding) strand of the gene sequence was included.

Table 3A. Table 3A contained all those sequences in BioCardia's subtracted libraries that matched sequences in GenBank's nr, EST_Human, and UniGene databases with an acceptable level of confidence. All the entries in the table representing the sense strand of their genes were grouped together and all those representing the antisense strand were grouped. A third group contained those entries whose strand could not be determined. Two complementary probes were designed for each member of this third group.

Table 3B and 3C. Table 3B and 3C contained all those sequences in the leukocyte expression subtracted library that did not match sequences in GenBank's nr, EST_Human, and UniGene databases with an acceptable level of confidence, but which had a high probability of representing real mRNA sequences. Sequences in Table 3B did not match anything in the databases above but matched regions of the human genome draft and were spatially clustered along it, suggesting that they were exons, rather than genomic DNA included in the library by chance. Sequences in Table 3C also aligned well to regions of the human genome draft, but the aligned regions were interrupted by genomic DNA, meaning they were likely to be spliced transcripts of multiple exon genes.

Table 3B lists 510 clones and Table 3C lists 48 clones that originally had no similarity with any sequence in the public databases. Blastn searches conducted after the initial filing have identified sequences in the public database with high similarity (E values less than $1e-40$) to the sequences determined for these clones. Table 3B contained 272 clones and Table 3C contained 25 clones that were found to have high similarity to sequences in dbEST. The sequences of the similar dbEST clones were

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used to design probes. Sequences from clones that contained no similar regions to any sequence in the database were used to design a pair of complementary probes.

Probes were designed from database sequences that had the highest similarity to each of the sequenced clones in Tables 3A, 3B, and 3C. Based on BLASTn searches the most similar database sequence was identified by locus number and the locus number was submitted to GenBank using batch Entrez (<http://www.ncbi.nlm.nih.gov/entrez/batchentrez.cgi?db=Nucleotide>) to obtain the sequence for that locus. The GenBank entry sequence was used because in most cases it was more complete or was derived from multi-pass sequencing and thus would likely have fewer errors than the single pass cDNA library sequences. When only UniGene cluster IDs were available for genes of interest, the respective sequences were extracted from the UniGene_unique database, build 137, downloaded from NCBI (<ftp://ncbi.nlm.nih.gov/repository/UniGene/>). This database contains one representative sequence for each cluster in UniGene.

Summary of BioCardia library clones used in probe design.

Table	Sense Strand	Antisense Strand	Strand Undetermined
Table 3A	3621	763	124
Table 3B	142	130	238
Table 3C	19	6	23
Totals	3782	899	385

Literature Searches

Example 2 describes searches of literature databases. We also searched for research articles discussing genes expressed only in leukocytes or involved in inflammation and particular disease conditions, including genes that were specifically expressed or down-regulated in a disease state. Searches included, but were not limited to, the following terms and various combinations of these terms: inflammation, atherosclerosis, rheumatoid arthritis, osteoarthritis, lupus, SLE, allograft, transplant, rejection, leukocyte, monocyte, lymphocyte, mononuclear, macrophage, neutrophil, eosinophil, basophil, platelet, congestive heart failure, expression, profiling, microarray, inflammatory bowel disease, asthma, RNA expression, gene expression, granulocyte.

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A UniGene cluster ID or GenBank accession number was found for each gene in the list. The strand of the corresponding sequence was determined, if possible, and the genes were divided into the three groups: sense (coding) strand, anti-sense strand, or strand unknown. The rest of the probe design process was carried out as described above for the sequences from the leukocyte subtracted expression library.

Database Mining

Database mining was performed as described in Example 2. In addition, the Library Browser at the NCBI UniGene web site (<http://www.ncbi.nlm.nih.gov/UniGene/lbrowse.cgi?ORG=Hs&DISPLAY=ALL>) was used to identify genes that are specifically expressed in leukocyte cell populations. All expression libraries available at the time were examined and those derived from leukocytes were viewed individually. Each library viewed through the Library Browser at the UniGene web site contains a section titled "Shown below are UniGene clusters of special interest only" that lists genes that are either highly represented or found only in that library. Only the genes in this section were downloaded from each library. Alternatively, every sequence in each library is downloaded and then redundancy between libraries is reduced by discarding all UniGene cluster IDs that are represented more than once.

A total of 439 libraries were downloaded, containing 35,819 genes, although many were found in more than one library. The most important libraries from the remaining set were separated and 3,914 genes remained. After eliminating all redundancy between these libraries and comparing the remaining genes to those listed in Tables 3A, 3B and 3C, the set was reduced to 2,573 genes in 35 libraries (listed below). From these, all genes in first 30 libraries were used to design probes. A random subset of genes was used from Library Lib.376, "Activated_T-cells_XX". From the last four libraries, a random subset of sequences listed as "ESTs, found only in this library" was used.

Library ID	Library Name	Category	No. of sequences before reduction	No. of sequences used on array*
Lib.2228	Human_leukocyte_MATCHMAKER_cDNA_Library	other/unclassified	4	3

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Lib.238	RA-MO-III (activated monocytes from RA patient)	Blood	2	1
Lib.242	Human_peripheral_blood_(Whole)_(Steve_Elledge)	Blood	4	2
Lib.2439	Subtracted_cDNA_libraries_from_human_Jurkat_cells	other/unclassified	4	1
Lib.323	Activated_T-cells_I	other/unclassified	19	3
Lib.327	Monocytes_stimulated_II	Blood	92	35
Lib.387	Macrophage_I	other/unclassified	84	24
Lib.409	Activated_T-cells_IV	other/unclassified	37	10
Lib.410	Activated_T-cells_VIII	other/unclassified	27	10
Lib.411	Activated_T-cells_V	other/unclassified	41	9
Lib.412	Activated_T-cells_XII	other/unclassified	29	12
Lib.413	Activated_T-cells_XI	other/unclassified	13	6
Lib.414	Activated_T-cells_II	other/unclassified	69	30
Lib.429	Macrophage_II	other/unclassified	56	24
Lib.4480	Homo_sapiens_rheumatoid_arthritis_fibroblast-like_synovial	other/unclassified	7	6
Lib.476	Macrophage_subtracted_(total_cDNA)	other/unclassified	11	1
Lib.490	Activated_T-cells_III	other/unclassified	9	5
Lib.491	Activated_T-cells_VII	other/unclassified	27	8
Lib.492	Activated_T-cells_IX	other/unclassified	16	5
Lib.493	Activated_T-cells_VI	other/unclassified	31	15
Lib.494	Activated_T-cells_X	other/unclassified	18	5
Lib.498	RA-MO-I (activated peripheral blood monocytes from RA patient)	Blood	2	1
Lib.5009	Homo_Sapiens_cDNA_Library_from_Peripheral_White_Blood_Cell	other/unclassified	3	3
Lib.6338	human_activated_B_lymphocyte	Tonsils	9	8
Lib.6342	Human_lymphocytes	other/unclassified	2	2
Lib.646	Human_leukocyte_(M.L.Markelov)	other/unclassified	1	1
Lib.689	Subtracted_cDNA_library_of_activated_B_lymphocyte	Tonsil	1	1
Lib.773	PMA-induced_HL60_cell_subtraction_library (leukemia)	other/unclassified	6	3
Lib.1367	cDNA_Library_from_rIL-2_activated_lymphocytes	other/unclassified	3	2
Lib.5018	Homo_sapiens_CD4+_T-cell_clone_HA1.7	other/unclassified	6	3
Lib.376	Activated_T-cells_XX	other/unclassified	999	119
Lib.669	NCI_CGAP_CLL1 (Lymphocyte)	Blood	353	81†
Lib.1395	NCI_CGAP_Sub6 (germinal center b-cells)	B cells germinal	389	100†
Lib.2217	NCI_CGAP_Sub7 (germinal center b-cells)	B cells germinal	605	200†
Lib.289	NCI_CGAP_GCB1 (germinal center b-cells)	Tonsil	935	200†
Total			3,914	939

* Redundancy of UniGene numbers between the libraries was eliminated.

† A subset of genes flagged as "Found only in this library" were taken.

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Angiogenesis Markers

215 sequences derived from an angiogenic endothelial cell subtracted cDNA library obtained from Stanford University were used for probe design. Briefly, using well known subtractive hybridization procedures, (as described in, e.g., US Patent Numbers 5,958,738; 5,589,339; 5,827,658; 5,712,127; 5,643,761; 5,565,340) modified to normalize expression by suppressing over-representation of abundant RNA species while increasing representation of rare RNA species, a library was produced that is enriched for RNA species (messages) that are differentially expressed between test (stimulated) and control (resting) HUVEC populations. The subtraction/suppression protocol was performed as described by the kit manufacturer (Clontech, PCR-select cDNA Subtraction Kit).

Pooled primary HUVECs (Clonetics) were cultured in 15% FCS, M199 (GibcoBRL) with standard concentrations of Heparin, Penicillin, Streptomycin, Glutamine and Endothelial Cell Growth Supplement. The cells were cultured on 1% gelatin coated 10 cm dishes. Confluent HUVECs were photographed under phase contrast microscopy. The cells formed a monolayer of flat cells without gaps. Passage 2-5 cells were used for all experiments. Confluent HUVECs were treated with trypsin/EDTA and seeded onto collagen gels. Collagen gels were made according to the protocol of the Collagen manufacturer (Becton Dickinson Labware). Collagen gels were prepared with the following ingredients: Rat tail collagen type I (Collaborative Biomedical) 1.5 mg/mL, mouse laminin (Collaborative Biomedical) 0.5 mg/mL, 10% 10X media 199 (Gibco BRL). 1N NaOH, 10 X PBS and sterile water were added in amounts recommended in the protocol. Cell density was measured by microscopy. 1.2×10^6 cells were seeded onto gels in 6-well, 35 mm dishes, in 5% FCS M199 media. The cells were incubated for 2 hrs at 37 C with 5% CO₂. The media was then changed to the same media with the addition of VEGF (Sigma) at 30ng/mL media. Cells were cultured for 36 hrs. At 12, 24 and 36 hrs, the cells were observed with phase contrast microscopy. At 36 hours, the cells were observed elongating, adhering to each other and forming lumen structures. At 12 and 24 hrs media was aspirated and refreshed. At 36 hrs, the media was aspirated, the cells were rinsed with PBS and then treated with Collagenase (Sigma) 2.5mg/mL PBS for 5 min with active agitation until the collagen gels were liquefied. The cells were then centrifuged at 4C, 2000g for 10 min. The supernatant was removed and the cells

Ten plates of resting, confluent, p4 HUVECs, were cultured with 15 % FCS in the M199 media described above. The media was aspirated and the cells were lysed with 1 mL Trizol and total RNA was prepared according to the Trizol protocol. mRNA was then isolated according to the micro-fast track mRNA isolation protocol from Invitrogen. This RNA served as the control RNA for the subtraction procedure.

A second hybridization involved pooling unhybridized sequences from the first hybridization together with the addition of supplemental driver cDNA. In this step, the expressed sequences enriched in the two tester pools following the initial hybridization can hybridize. Hybrids resulting from the hybridization between members of each of the two tester pools are then recovered by amplification in a polymerase chain reaction (PCR) using primers specific for the unique adapters. Again, sequences originating in a tester pool that form hybrids with components of the driver pool are not amplified. Hybrids resulting between members of the same tester pool are eliminated by the formation of "panhandles" between their common 5' and 3' ends. This process is illustrated schematically in Figure 3. The subtraction was done in both directions, producing two libraries, one with clones that are upregulated in tube-formation and one with clones that are down-regulated in the process.

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The resulting PCR products representing partial cDNAs of differentially expressed genes were then cloned (i.e., ligated) into an appropriate vector according to the manufacturer's protocol (pGEM-Teasy from Promega) and transformed into competent bacteria for selection and screening. Colonies (2180) were picked and cultured in LB broth with 50ug/mL ampicillin at 37C overnight. Stocks of saturated LB + 50 ug/mL ampicillin and 15% glycerol in 96-well plates were stored at -80C. Plasmid was prepared from 1.4mL saturated LB broth containing 50 ug/mL ampicillin. This was done in a 96 well format using commercially available kits according to the manufacturer's recommendations (Qiagen 96-turbo prep).

2 probes to represent 22 of these sequences required, therefore, a total of 237 probes were derived from this library.

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Viral genes.

Several viruses may play a role in a host of disease including inflammatory disorders, atherosclerosis, and transplant rejection. The table below lists the viral genes represented by oligonucleotide probes on the microarray. Low-complexity regions in the sequences were masked using RepeatMasker before using them to design probes.

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Virus	Gene Name	Genome Location
Adenovirus, type 2 Accession #J01917	E1a	1226..1542
	E1b_1	3270...3503
	E2a_2	complement(24089..25885)
	E3-1	27609..29792
	E4 (last exon at 3'-end)	complement(33193..32802)
	IX	3576..4034
	Iva2	complement(4081..5417)
	DNA Polymerase	complement(5187..5418)
Cytomegalovirus (CMV) Accession #X17403	HCMVTRL2 (IRL2)	1893..2240
	HCMVTRL7 (IRL7)	complement(6595..6843)
	HCMVUL21	complement(26497..27024)
	HCMVUL27	complement(32831..34657)
	HCMVUL33	43251..44423
	HCMVUL54	complement(76903..80631)
	HCMVUL75	complement(107901..110132)
	HCMVUL83	complement(119352..121037)
	HCMVUL106	complement(154947..155324)
	HCMVUL109	complement(157514..157810)
	HCMVUL113	161503..162800
	HCMVUL122	complement(169364..170599)
	HCMVUL123 (last exon at 3'-end)	complement(171006..172225)
	HCMVUS28	219200..220171
Epstein-Barr virus (EBV) Accession # NC_001345	Exon in EBNA-1 RNA	67477..67649
	Exon in EBNA-1 RNA	98364..98730
	BRLF1	complement(103366..105183)
	BZLF1 (first of 3 exons)	complement(102655..103155)
	BMLF1	complement(82743..84059)
	BALF2	complement(161384..164770)
Human Herpesvirus 6 (HHV6) Accession #NC_001664	U16/U17	complement(26259..27349)
	U89	complement(133091..135610)
	U90	complement(135664..135948)
	U86	complement(125989..128136)
	U83	123528..123821
	U22	complement(33739..34347)
	DR2 (DR2L)	791..2653
	DR7 (DR7L)	5629..6720
	U95	142941..146306
	U94	complement(141394..142866)
	U39	complement(59588..62080)
	U42	complement(69054..70598)
	U81	complement(121810..122577)

Strand Selection

It was necessary to design sense oligonucleotide probes because the labeling and hybridization protocol to be used with the microarray results in fluorescently-labeled antisense cRNA. All of the sequences we selected to design probes could be divided into three categories:

- (1) Sequences known to represent the sense strand
- (2) Sequences known to represent the antisense strand
- (3) Sequences whose strand could not be easily determined from their descriptions

It was not known whether the sequences from the leukocyte subtracted expression library were from the sense or antisense strand. GenBank sequences are reported with sequence given 5' to 3', and the majority of the sequences we used to design probes came from accession numbers with descriptions that made it clear whether they represented sense or antisense sequence. For example, all sequences containing "mRNA" in their descriptions were understood to be the sequences of the sense mRNA, unless otherwise noted in the description, and all IMAGE Consortium clones are directionally cloned and so the direction (or sense) of the reported sequence can be determined from the annotation in the GenBank record.

For accession numbers representing the sense strand, the sequence was downloaded and masked and a probe was designed directly from the sequence. These probes were selected as close to the 3' end as possible. For accession numbers representing the antisense strand, the sequence was downloaded and masked, and a probe was designed complementary to this sequence. These probes were designed as close to the 5' end as possible (i.e., complementary to the 3' end of the sense strand).

Minimizing Probe Redundancy.

Multiple copies of certain genes or segments of genes were included in the sequences from each category described above, either by accident or by design. Reducing redundancy within each of the gene sets was necessary to maximize the number of unique genes and ESTs that could be represented on the microarray.

Three methods were used to reduce redundancy of genes, depending on what information was available. First, in gene sets with multiple occurrences of one or more

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UniGene numbers, only one occurrence of each UniGene number was kept. Next, each gene set was searched by GenBank accession numbers and only one occurrence of each accession number was conserved. Finally, the gene name, description, or gene symbol were searched for redundant genes with no UniGene number or different accession numbers. In reducing the redundancy of the gene sets, every effort was made to conserve the most information about each gene.

We note, however, that the UniGene system for clustering submissions to GenBank is frequently updated and UniGene cluster IDs can change. Two or more clusters may be combined under a new cluster ID or a cluster may be split into several new clusters and the original cluster ID retired. Since the lists of genes in each of the gene sets discussed were assembled at different times, the same sequence may appear in several different sets with a different UniGene ID in each.

Sequences from Table 3A were treated differently. In some cases, two or more of the leukocyte subtracted expression library sequences aligned to different regions of the same GenBank entry, indicating that these sequences were likely to be from different exons in the same gene transcript. In these cases, one representative library sequence corresponding to each presumptive exon was individually listed in Table 3A.

Compilation.

After redundancy within a gene set was sufficiently reduced, a table of approximately 8,000 unique genes and ESTs was compiled in the following manner. All of the entries in Table 3A were transferred to the new table. The list of genes produced by literature and database searches was added, eliminating any genes already contained in Table 3A. Next, each of the remaining sets of genes was compared to the table and any genes already contained in the table were deleted from the gene sets before appending them to the table.

	<u>Probes</u>
BioCardia Subtracted Leukocyte Expression Library	
Table 3A	4,872
Table 3B	796
Table 3C	85
Literature Search Results	494

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Database Mining	1,607
Viral genes	
a. CMV	14
b. EBV	6
c. HHV 6	14
d. Adenovirus	8
Angiogenesis markers: 215, 22 of which needed two probes	237
<i>Arabidopsis thaliana</i> genes	10
Total sequences used to design probes	8,143

Example 21-Design of oligonucleotide probes

This section describes the design of four oligonucleotide probes using Array Designer Ver 1.1 (Premier Biosoft International, Palo Alto, CA).

Clone 40H12

Clone 40H12 was sequenced and compared to the nr, dbEST, and UniGene databases at NCBI using the BLAST search tool. The sequence matched accession number NM_002310, a 'curated RefSeq project' sequence, see Pruitt et al. (2000) Trends Genet. 16:44-47, encoding leukemia inhibitory factor receptor (LIFR) mRNA with a reported E value of zero. An E value of zero indicates there is, for all practical purposes, no chance that the similarity was random based on the length of the sequence and the composition and size of the database. This sequence, cataloged by accession number NM_002310, is much longer than the sequence of clone 40H12 and has a poly-A tail. This indicated that the sequence cataloged by accession number NM_002310 is the sense strand and a more complete representation of the mRNA than the sequence of clone 40H12, especially at the 3' end. Accession number "NM_002310" was included in a text file of accession numbers representing sense strand mRNAs, and sequences for the sense strand mRNAs were obtained by uploading a text file containing desired accession numbers as an Entrez search query using the Batch Entrez web interface and saving the results locally as a FASTA file. The following sequence was obtained, and the region of alignment of clone 40H12 is outlined:

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CTCTCTCCAGAACGTGTCTCTGCTGCAAGGCACCGGGCCCTTTTCGCTCTGCAGAACTGC
ACTTGCAAGACCATTATCAACTCCTAATCCCAGCTCAGAAAGGGAGCCTCTGCGACTCAT
TCATCGCCCTCCAGGACTGACTGCATTGCACAGATGATGGATATTTACGTATGTTTGAAA
CGACCATCCTGGATGGTGGACAATAAAAGAATGAGGACTGCTTCAAATTTCCAGTGGCTG
TTATCAACATTTTATTCTTCTATATCTAATGAATCAAGTAAATAGCCAGAAAAAGGGGGCT
CCTCATGATTTGAAGTGTGTAACATAAATTTGCAAGTGTGGAAGTGTCTTGGAAGCA
CCCTCTGGAACAGGCCGTGGTACTGATTATGAAGTTTGCATTGAAAACAGGTCCCGTTCT
TGTTATCAGTTGGAGAAAACCAGTATTAAAATTCAGCTCTTTCACATGGTGATTATGAA
ATAACAATAAATTTCTCTACATGATTTTGGAAGTTCTACAAGTAAATTCACACTAAATGAA
CAAAACGTTTTCCTTAATTCAGATACTCCAGAGATCTTGAATTTGTCTGCTGATTTCTCA
ACCTCTACATTATACCTAAAGTGAACGACAGGGGTTTCAGTTTTTCCACACCGCTCAAAT
GTTATCTGGGAAATTAAAGTTCTACGTAAAGAGAGTATGGAGCTCGTAAAATTAGTGACC
CACAACACAACCTCTGAATGGCAAAGATACACTTCATCACTGGAGTTGGGCCTCAGATATG
CCCTTGGAATGTGCCATTTCATTTTGTGGAAATTAGATGCTACATTGACAATCTTCATTTT
TCTGGTCTCGAAGAGTGGAGTGAAGTGGAGCCCTGTGAAGAACATTTCTTGATACCTGAT
TCTCAGACTAAGGTTTTTCTCAAGATAAAGTGATACTTGTAGGCTCAGACATAACATTT
TGTTGTGTGAGTCAAGAAAAAGTGTATCAGCACTGATTGGCCATACAACTGCCCCCTTG
ATCCATCTTGATGGGGAAAATGTTGCAATCAAGATTCGTAATATTTCTGTTTCTGCAAGT
AGTGGAACAAATGTAGTTTTTACAACCGAAGATAACATATTTGGAACCGTTATTTTTGCT
GGATATCCACCAGATACTCCTCAACAACCTGAATTGTGAGACACATGATTTAAAGAAAT
ATATGTAGTTGGAATCCAGGAAGGGTGACAGCGTTGGTGGGCCCACGTGCTACAAGCTAC
ACTTTAGTTGAAAGTTTTTTCAGGAAAATATGTTAGACTTAAAAGAGCTGAAGCACCTACA
AACGAAAGCTATCAATTATTATTTCAAATGCTTCCAAATCAAGAAATATATAATTTTACT
TTGAATGCTCACAATCCGCTGGGTGATCACAATCAACAATTTTAGTTAATATAACTGAA
AAAGTTTATCCCCATACTCCTACTTCATTCAAAGTGAAGGATATTAATTCAACAGCTGTT
AAACTTTCTTGGCATTTACCAGGCAACTTTGCAAAGATTAATTTTTTATGTGAAATTGAA
ATTAAGAAATCTAATTCAGTACAAGAGCAGCGGAATGTCACAATCAAAGGAGTAGAAAAAT
TCAAGTTATCTTGTTGCTCTGGACAAGTTAAATCCATACACTCTATATACTTTTCGGATT
CGTTGTTCTACTGAACTTTCTGGAAATGGAGCAAAATGGAGCAATAAAAAACAACATTTA
ACAACAGAAGCCAGTCCTTCAAAGGGGCCTGATACTTGGAGAGAGTGGAGTTCTGATGGA
AAAAATTTAATAATCTATTGGAAGCCTTTACCCATTAATGAAGCTAATGGAAAAATACTT

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TCCTACAATGTATCGTGTTTCATCAGATGAGGAAACACAGTCCCTTTCTGAAATCCCTGAT
CCTCAGCACAAAGCAGAGATACGACTTGATAAGAATGACTACATCATCAGCGTAGTGGCT
AAAAATTCTGTGGGCTCATCACCACCTTCCAAAATAGCGAGTATGGAAATTCCAAATGAT
GATCTCAAAATAGAACAAGTTGTTGGGATGGGAAAGGGGATTCTCCTCACCTGGCATTAC
GACCCCAACATGACTTGCGACTACGTCATTAAGTGGTGTAACCTCGTCTCGGTGCGAACCA
TGCCTTATGGACTGGAGAAAAGTTCCCTCAAACAGCACTGAAACTGTAATAGAATCTGAT
GAGTTTCGACCAGGTATAAGATATAATTTTTTCTGTATGGATGCAGAAATCAAGGATAT
CAATTATTACGCTCCATGATTGGATATATAGAAGAATTGGCTCCCATTGTTGCACCAAAT
TTTACTGTTGAGGATACTTCTGCAGATTCGATATTAGTAAAATGGGAAGACATTCTGTG
GAAGAACTTAGAGGCTTTTTAAGAGGATATTTGTTTTACTTTGGAAAAGGAGAAAGAGAC
ACATCTAAGATGAGGGTTTTAGAAATCAGGTCGTTCTGACATAAAAAGTTAAGAATATTACT
GACATATCCCAGAAGACACTGAGAATTGCTGATCTTCAAGGTAAAAACAAGTTACCACCTG
GTCTTGCGAGCCTATACAGATGGTGGAGTGGGCCCGGAGAAGAGTATGTATGTGGTGACA
AAGGAAAATTCTGTGGGATTAATTATTGCCATTCTCATCCCAGTGGCAGTGGCTGTCATT
GTTGGAGTGGTGACAAGTATCCTTTGCTATCGGAAACGAGAATGGATTAAAGAAACCTTC
TACCCTGATATTCCAAATCCAGAAAACCTGTAAAGCATTACAGTTTCAAAGAGTGTCTGT
GAGGGAAGCAGTGCTCTTAAACATTGGAAATGAATCCTTGTAACCCCAAATAATGTTGAG
GTTCTGGAAACTCGATCAGCATTTCTTAAATAGAAGATACAGAAATAATTTCCCCAGTA
GCTGAGCGTCCTGAAGATCGCTCTGATGCAGAGCCTGAAAACCATGTGGTTGTGTCCTAT
TGTCACCCCATCATTGAGGAAGAAATACCAAACCCAGCCGCAGATGAAGCTGGAGGGACT
GCACAGGTTATTTACATTGATGTTTCAGTCGATGTATCAGCCTCAAGCAAACCCAGAAGAA
GAACAAGAAAATGACCCTGTAGGAGGGGCAGGCTATAAGCCACAGATGCACCTCCCCATT
AATTCTACTGTGGAAGATATAGCTGCAGAAGAGGACTTAGATAAAAACCTGCGGGTTACAGA
CCTCAGGCCAATGTAAATACATGGAATTTAGTGTCTCCAGACTCTCCTAGATCCATAGAC
AGCAACAGTGAGATTGTCTCATTTGGAAGTCCATGCTCCATTAATTCCCGACAATTTTG
ATTCCTCCTAAAGATGAAGACTCTCCTAAATCTAATGGAGGAGGGTGGTCCTTTACAAAC
TTTTTTCAGAACAAACCAAACGATTAAACAGTGTACCCGTGTCACTTCAGTCAGCCATCTC
AATAAGCTCTTACTGCTAGTGTGCTACATCAGCACTGGGCATTCTTGAGGGGATCCTGT
GAAGTATTGTTAGGAGGTGAACCTTCACTACATGTTAAGTTACACTGAAAGTTTCATGTGCT
TTTAATGTAGTCTAAAAGCCAAAGTATAGTGACTCAGAATCCTCAATCCACAAAACCTCAA
GATTGGGAGCTCTTTGTGATCAAGCCAAAGAATTCATGTACTCTACCTTCAAGAAGCA
TTTCAAGGCTAATACCTACTTGTACGTACATGTAAACAAATCCCGCCGCAACTGTTTTTC

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TGTTCTGTTGTTTGTGGTTTTCTCATATGTATACTTGGTGGAAATTGTAAGTGGATTTGCA
 GGCCAGGGAGAAAAATGTCCAAGTAACAGGTGAAGTTTATTTGCCTGACGTTTACTCCTTT
 CTAGATGAAAACCAAGCACAGATTTTAAACTTCTAAGATTATTCTCCTCTATCCACAGC
 ATTCACAAAAATTAATATAATTTTTAATGTAGTGACAGCGATTTAGTGTTTTGTGTTGATA
 AAGTATGCTTATTTCTGTGCCTACTGTATAATGGTTATCAAACAGTTGTCTCAGGGGTAC
 AAACTTTGAAAACAAGTGTGACACTGACCAGCCCAAATCATAATCATGTTTCTTGCTGT
 GATAGGTTTGTGCTTGCCTTTTCATTATTTTTTAGCTTTTATGCTTGCTTCCATTATTTCA
 GTTGGTTGCCCTAATATTTAAATTTACACTTCTAAGACTAGAGACCCACATTTTTTAA
 AATCATTTTATTTTGTGATACAGTGACAGCTTTATATGAGCAAATTCAATATTATTCATA
 AGCATGTAATCCAGTGACTTACTATGTGAGATGACTACTAAGCAATATCTAGCAGCGTT
 AGTTCCATATAGTTCTGATTGGATTTCGTTCTCCTGAGGAGACCATGCCGTTGAGCTTG
 GCTACCCAGGCAGTGGTGATCTTTGACACCTTCTGGTGGATGTTCTCCCACTCATGAGT
 CTTTTCATCATGCCACATTATCTGATCCAGTCCTCACATTTTTTAAATATAAACTAAAGA
 GAGAATGCTTCTTACAGGAACAGTTACCCAAGGGCTGTTTCTTAGTAACGTGCATAAACT
 GATCTGGATCCATGGGCATACCTGTGTTTCGAGGTGCAGCAATTGCTTGCTGAGCTGTGCA
 GAATTGATTGCCTTCAGCACAGCATCCTCTGCCCACCCTTGTTTCTCATAAGCGATGTCT
 GGAGTGATTGTGGTTCTTGGAAGCAGAAGGAAAAACTAAAAAGTGATCTTGTATTTT
 CCTGCCCCTCAGGTTGCCTATGTATTTTACCTTTTCATATTTAAGGCAAAAGTACTTGAA
 AATTTTAAGTGTCCGAATAAGATATGTCTTTTTTGTGTTGTTTTTTTTGTTGGTTGTTT
 TTTTTTATCATCTGAGATTCTGTAATGTATTTGCAATAATGGATCAATTAATTTTTTTT
 GAAGCTCATATTGTATCTTTTTTAAAAACCATGTTGTGGAAAAAGCCAGAGTGACAAGTG
 ACAAATCTATTTAGGAACTCTGTGTATGAATCCTGATTTTAACTGCTAGGATTCAGCTA
 AATTTCTGAGCTTTATGATCTGTGGAAATTTGGAATGAAATCGAATTCATTTGTACATA
 CATAGTATATTAAACTATATAATAGTTTCATAGAAATGTTTCAGTAATGAAAAATATATC
 CAATCAGAGCCATCCCGAAAAAATAAAAAA (SEQ ID No. : 8827)

The FASTA file, including the sequence of NM_002310, was masked using the RepeatMasker web interface (Smit, AFA & Green, P RepeatMasker at <http://ftp.genome.washington.edu/RM/RepeatMasker.html>, Smit and Green). Specifically, during masking, the following types of sequences were replaced with "N's": SINE/MIR & LINE/L2, LINE/L1, LTR/MaLR, LTR/Retroviral, Alu, and other low

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CTCTCTCCCAGAACGTGTCTCTGCTGCAAGGCACCGGGCCCTTTCGCTCTGCAGAACTG
CACTTGCAAGACCATTATCAACTCCTAATCCCAGCTCAGAAAGGGAGCCTCTGCGACTC
ATTCATCGCCCTCCAGGACTGACTGCATTGCACAGATGATGGATATTTACGTATGTTTG
AAACGACCATCCTGGATGGTGGACAATAAAAGAATGAGGACTGCTTCAAATTTCCAGTG
GCTGTTATCAACATTTATTCTTCTATATCTAATGAATCAAGTAAATAGCCAGAAAAAGG
GGGCTCCTCATGATTTGAAGTGTGTAACATAAATTTGCAAGTGTGGAACGTGTTCTTG
AAAGCACCCCTCTGGAACAGGCCGTGGTACTGATTATGAAGTTTGATTGAAAACAGGTC
CCGTTCTTGTTATCAGTTGGAGAAAACCAGTATTAATAATCCAGCTCTTTCACATGGTG
ATTATGAAATAACAATAAATTCTCTACATGATTTTGGAAGTTCTACAAGTAAATTCACA
CTAAATGAACAAAACGTTTCCTTAATTCCAGATACTCCAGAGATCTTGAATTTGTCTGC
TGATTTCTCAACCTCTACATTATACCTAAAGTGGAACGACAGGGGTTTCAGTTTTTCCAC
ACCGCTCAAATGTTATCTGGGAAATTAAAGTTCTACGTAAAGAGAGTATGGAGCTCGTA
AAATTAGTGACCCACAACACAACCTCTGAATGGCAAAGATACACTTCATCACTGGAGTTG
GGCCTCAGATATGCCCTTGGAATGTGCCATTCATTTTGTGGAAATTAGATGCTACATTG
ACAATCTTCATTTTTCTGGTCTCGAAGAGTGGAGTGAAGTGGAGCCCTGTGAAGAACATT
TCTTGATACCTGATTCTCAGACTAAGGTTTTTCTCAAGATAAAGTGATACTTGTAGG
CTCAGACATAACATTTTTGTTGTGTGAGTCAAGAAAAAGTGTTATCAGCACTGATTGGCC
ATACAACTGCCCCCTTGATCCATCTTGATGGGGAAAATGTTGCAATCAAGATTCGTAAT
ATTTCTGTTTCTGCAAGTAGTGGAACAAATGTAGTTTTTTACAACCGAAGATAACATATT
TGGAACCGTTATTTTTGCTGGATATCCACCAGATACTCCTCAACAACTGAATTGTGAGA
CACATGATTTAAAAGAAATTATATGTAGTTGGAATCCAGGAAGGGTGACAGCGTTGGTG
GGCCACGTGCTACAAGCTACACTTTAGTTGAAAGTTTTTCAGGAAAATATGTTAGACT
TAAAAGAGCTGAAGCACCTACAAACGAAAGCTATCAATTATTATTTCAAATGCTTCCAA
ATCAAGAAATATATAATTTTACTTTGAATGCTCACAATCCGCTGGGTGATCACAATCA
ACAATTTTAGTTAATATAACTGAAAAAGTTTATCCCCATACTCCTACTTCATTCAAAGT
GAAGGATATTAATTCAACAGCTGTTAACTTTCTTGGCATTACCAGGCAACTTTGCAA
AGATTAATTTTTTATGTGAAATTGAAATTAAGAAATCTAATTCAGTACAAGAGCAGCGG
AATGTACAATCAAAGGAGTAGAAAATTCAAGTTATCTTGTTGCTCTGGACAAGTTAA
TCCATACACTCTATATACTTTTCGGATTTCGTTGTTCTACTGAAACTTTCTGGAAATGG

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GCAAATGGAGCAATAAAAAACAACATTTAACAACAGAAGCCAGTCCTTCAAAGGGGCCT
GATACTTGGAGAGAGTGGAGTTCTGATGGAAAAAATTTAATAATCTATTGGAAGCCTTT
ACCCATTAATGAAGCTAATGGAAAAATACTTTCCTACAATGTATCGTGTTTCATCAGATG
AGGAAACACAGTCCCCTTTCTGAAATCCCTGATCCTCAGCACAAAGCAGAGATACGACTT
GATAAGAATGACTACATCATCAGCGTAGTGGCTAAAAATTCTGTGGGCTCATCACCACC
TTCCAAAATAGCGAGTATGGAAATTCCAAATGATGATCTCAAAATAGAACAAGTTGTTG
GGATGGGAAAGGGGATTCTCCTCACCTGGCATTACGACCCCAACATGACTTGCGACTAC
GTCATTAAGTGGTGTAACCTCGTCTCGGTGGAACCATGCCTTATGGACTGGAGAAAAGT
TCCCTCAAACAGCACTGAAACTGTAATAGAATCTGATGAGTTTCGACCAGGTATAAGAT
ATAATTTTTTCTGTATGGATGCAGAAATCAAGGATATCAATTATTACGCTCCATGATT
GGATATATAGAAGAATTGGCTCCCATTTGTTGCACCAAATTTTACTGTTGAGGATACTTC
TGCAGATTCGATATTAGTAAAATGGGAAGACATTCCTGTGGAAGAACTTAGAGGCTTTT
TAAGAGGATATTTGTTTTACTTTGGAAAAGGAGAAAGAGACACATCTAAGATGAGGGTT
TTAGAATCAGGTCGTTCTGACATAAAAGTTAAGAATATTACTGACATATCCCAGAAGAC
ACTGAGAATTGCTGATCTTCAAGGTAAAAACAAGTTACCACCTGGTCTTGCGAGCCTATA
CAGATGGTGGAGTGGGCCCGGAGAAGAGTATGTATGTGGTGACAAAGGAAAATTCTGTG
GGATTAATTATTGCCATTCTCATCCCAGTGGCAGTGGCTGTATTGTTGGAGTGGTGAC
AAGTATCCTTTGCTATCGGAAACGAGAATGGATTAAAGAAACCTTCTACCCTGATATTC
CAAATCCAGAAAACCTGTAAAGCATTACAGTTTCAAAGAGTGTCTGTGAGGGAAGCAGT
GCTCTTAAACATTGGAAATGAATCCTTGTACCCCAAATAATGTTGAGGTTCTGGAAAC
TCGATCAGCATTTCCTAAATAGAAGATACAGAAATAATTTCCCAGTAGCTGAGCGTC
CTGAAGATCGCTCTGATGCAGAGCCTGAAAACCATGTGGTTGTGTCTATTGTCCACCC
ATCATTGAGGAAGAAATACCAAACCCAGCCGAGATGAAGCTGGAGGGACTGCACAGGT
TATTTACATTGATGTTTCAGTCGATGTATCAGCCTCAAGCAAAACCAGAAGAACAAG
AAAATGACCCTGTAGGAGGGGCAGGCTATAAGCCACAGATGCACCTCCCCATTAATTCT
ACTGTGGAAGATATAGCTGCAGAAGAGGACTTAGATAAACTGCGGGTTACAGACCTCA
GGCCAATGTAAATACATGGAATTTAGTGTCTCCAGACTCTCCTAGATCCATAGACAGCA
ACAGTGAGATTGTCTCATTTGGAAGTCCATGCTCCATTAATTCCCGACAATTTTGTATT
CCTCCTAAAGATGAAGACTCTCCTAAATCTAATGGAGGAGGGTGGTCTTTACAACTT
TTTTCAGAACAAACCAAACGATTAACAGTGTACCGTGTCACTTCAGTCAGCCATCTCA
ATAAGCTCTTACTGCTAGTGTGTACATCAGCACTGGGCATTCTTGAGGGGATCCTGT
GAAGTATTGTTAGGAGGTGAACTTCACTACATGTTAAGTTACACTGAAAGTTCATGTGC

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A SEO ID No. : 8828

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The length of this sequence was determined using batch, automated computational methods and the sequence, as sense strand, its length, and the desired location of the probe sequence near the 3' end of the mRNA was submitted to Array Designer Ver 1.1 (Premier Biosoft International, Palo Alto, CA). Search quality was set at 100%, number of best probes set at 1, length range set at 50 base pairs, Target T_m set at 75 C. degrees plus or minus 5 degrees, Hairpin max deltaG at 6.0 -kcal/mol., Self dimmer max deltaG at 6.0 -kcal/mol, Run/repeat (dinucleotide) max length set at 5, and Probe site minimum overlap set at 1. When none of the 49 possible probes met the criteria, the probe site would be moved 50 base pairs closer to the 5' end of the sequence and resubmitted to Array Designer for analysis. When no possible probes met the criteria, the variation on melting temperature was raised to plus and minus 8 degrees and the number of identical basepairs in a run increased to 6 so that a probe sequence was produced.

In the sequence above, using the criteria noted above, Array Designer Ver 1.1 designed a probe corresponding to oligonucleotide number 2280 in Table 8 and is indicated by underlining in the sequence above. It has a melting temperature of 68.4 degrees Celsius and a max run of 6 nucleotides and represents one of the cases where the criteria for probe design in Array Designer Ver 1.1 were relaxed in order to obtain an oligonucleotide near the 3' end of the mRNA (Low melting temperature was allowed).

Clone 463D12

Clone 463D12 was sequenced and compared to the nr, dbEST, and UniGene databases at NCBI using the BLAST search tool. The sequence matched accession number AI184553, an EST sequence with the definition line "qd60a05.x1 Soares_testis_NHT Homo sapiens cDNA clone IMAGE:1733840 3' similar to gb:M29550 PROTEIN PHOSPHATASE 2B CATALYTIC SUBUNIT 1 (HUMAN);, mRNA sequence." The E value of the alignment was 1.00×10^{-118} . The GenBank sequence begins with a poly-T region, suggesting that it is the antisense strand, read 5' to 3'. The beginning of this sequence is complementary to the 3' end of the mRNA sense strand. The accession number for this sequence was included in a text file of accession numbers representing antisense sequences. Sequences for antisense strand mRNAs were obtained by uploading a text file containing desired accession numbers as an Entrez

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search query using the Batch Entrez web interface and saving the results locally as a FASTA file. The following sequence was obtained, and the region of alignment of clone 463D12 is outlined:

```
TTTTTTTTTTTTTTCTTAAATAGCATTTATTTTCTCTCAAAAAGCCTATTATGTACTAA
CAAGTGTTCTCTAAATTAGAAAGGCATCACTACTAAAATTTTATACATATTTTTTTATA
TAAGAGAAGGAATATTGGGTTACAATCTGAATTTCTCTTTATGATTTCTCTTAAAGTAT
AGAACAGCTATTAAAATGACTAATATTGCTAAAATGAAGGCTACTAAATTTCCCAAGA
ATTTTCGGTGGAATGCCCAAAAATGGTGTTAAGATATGCAGAAGGGCCCATTTCAAGCAA
AGCAATCTCTCCACCCCTTCATAAAAGATTTAAGCTAAAAAAAAAAAAAAAAAGAA[GAAA]
[ATCCAACAGCTGAAGACATTGGGCTATTTATAAATCTTCTCCAGTCCCCCAGACAGCC]
[TCACATGGGGGCTGTAAACAGCTAACTAAAATATCTTTGAGACTCTTATGTCCACACCC]
[ACTGACACAAGGAGAGCTGTAACCACAGTGAACTAGACTTTGCTTTCCTTTAGCAAGT]
[ATGTGCCTATGATAGTAACTGGAGTAAATGTAACA]GTAATAAAACAAATTTTTTTTAA
AAATAAAAATTATACCTTTTTCTCCAACAAACGGTAAAGACCACGTGAAGACATCCATA
AAATTAGGCAACCAGTAAAGATGTGGAGAACCAGTAAACTGTCGAAATTCATCACATTA
TTTTCATACTTTAATACAGCAGCTTTAATTATTGGAGAACATCAAAGTAATTAGGTGCC
GAAAAACATTGTTATTAATGAAGGGAACCCCTGACGTTTGACCTTTTCTGTACCATCTA
TAGCCCTGGACTTGA (SEQ ID No. : 8829)
```

The FASTA file, including the sequence of AA184553, was then masked using the RepeatMasker web interface, as shown below. The region of alignment of clone 463D12 is outlined.

```
TTTTTTTTTTTTTTCTTAAATAGCATTTATTTTCTCTCAAAAAGCCTATTATGTACTAA
CAAGTGTTCTCTAAATTAGAAAGGCATCACTACNNNNNNNNNNNNNNNNNNNNNNNNNNNN
NNNGAGAAGGAATATTGGGTTACAATCTGAATTTCTCTTTATGATTTCTCTTAAAGTAT
AGAACAGCTATTAAAATGACTAATATTGCTAAAATGAAGGCTACTAAATTTCCCAAGA
ATTTTCGGTGGAATGCCCAAAAATGGTGTTAAGATATGCAGAAGGGCCCATTTCAAGCAA
AGCAATCTCTCCACCCCTTCATAAAAGATTTAAGCTAAAAAAAAAAAAAAAAAGAA[GAAA]
[ATCCAACAGCTGAAGACATTGGGCTATTTATAAATCTTCTCCAGTCCCCCAGACAGCC]
[TCACATGGGGGCTGTAAACAGCTAACTAAAATATCTTTGAGACTCTTATGTCCACACCC]
```

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ACTGACACAAGGAGAGCTGTAACCACAGTGAACTAGACTTTGCTTTCCTTTAGCAAGT
ATGTGCCTATGATAGTAACTGGAGTAAATGTAACAGNNNNNNNNNNNNNNNNNNNNNNNNNN
 NNNNNNNNNNNNNNNNNCCTTTTCTCCAACAAACGGTAAAGACCACGTGAAGACATCCATA
 AAATTAGGCAACCAGTAAAGATGTGGAGAACCAGTAAACTGTCGAAATTCATCACATTA
 TTTTCATACTTTAATACAGCAGCTTTAATTATTGGAGAACATCAAAGTAATTAGGTGCC
 GAAAAACATTGTTATTAATGAAGGGAACCCCTGACGTTTGACCTTTTCTGTACCATCTA
 TAGCCCTGGACTTGA Masked version of 463D12 sequence. (SEQ ID
 NO:8830)

The sequence was submitted to Array Designer as described above, however, the desired location of the probe was indicated at base pair 50 and if no probe met the criteria, moved in the 3' direction. The complementary sequence from Array Designer was used, because the original sequence was antisense. The oligonucleotide designed by Array Designer corresponds to oligonucleotide number 4342 in Table 8 and is complementary to the underlined sequence above. The probe has a melting temperature of 72.7 degrees centigrade and a max run of 4 nucleotides.

Clone 72D4

Clone 72D4 was sequenced and compared to the nr, dbEST, and UniGene databases at NCBI using the BLAST search tool. No significant matches were found in any of these databases. When compared to the human genome draft, significant alignments were found to three consecutive regions of the reference sequence NT_008060, as depicted below, suggesting that the insert contains three spliced exons of an unidentified gene.

Residue numbers on clone 72D4 sequence	Matching residue numbers on NT_008060
1 – 198	478646 – 478843
197 – 489	479876 – 480168
491 – 585	489271 – 489365

Because the reference sequence contains introns and may represent either the coding or noncoding strand for this gene, BioCardia's own sequence file was used to design the oligonucleotide. Two complementary probes were designed to ensure that the

sense strand was represented. The sequence of the insert in clone 72D4 is shown below, with the three putative exons outlined.

```

CAGGTCACACAGCACATCAGTGGCTACATGTGAGCTCAGACCTGGGTCTGCT
GCTGTCTGTCTTCCCAATATCCATGACCTTGACTGATGCAGGTGTCTAGGGAT
ACGTCCATCCCCGTCCTGCTGGAGCCCAGAGCACGGAAGCCTGGCCCTCCGA
GGAGACAGAAGGGAGTGTCTGGACACCATGACGAGAGCTTGGCAGAATAAAT
AACTTCTTTAAACAATTTTACGGCATGAAGAAATCTGGACCAGTTTATTAAAT
GGGATTTCTGCCACAAACCTTGGAAGAATCACATCATCTTANNCCCAAGTGA
AAACTGTGTTGCGTAACAAAGAACATGACTGCGCTCCACACATACATCATTG
CCCGGCGAGGCGGGACACAAGTCAACGACGGAACACTTGAGACAGGCCTAC
AACTGTGCACGGGTCAGAAGCAAGTTTAAAGCCATACTTGCTGCAGTGAGACT
ACATTTCTGTCTATAGAAGATACTGACTTGATCTGTTTTTCAGCTCCAGTTC
CCAGATGTGCGTGTGTGGTCCCCAAGTATCACCTTCCAATTTCTGGGAGCA
GTGCTCTGGCCG GATCCTTGCCGCGCGGATAAAAAC (SEQ ID NO.: 8445)

```

The sequence was submitted to RepeatMasker, but no repetitive sequences were found. The sequence shown above was used to design the two 50-mer probes using Array Designer as described above. The probes are shown in bold typeface in the sequence depicted below. The probe in the sequence is oligonucleotide number 6415 (SEQ ID NO.: 6415) in Table 8 and the complementary probe is oligonucleotide number 6805 (SEQ ID NO.: 6805).

```

CAGGTCACACAGCACATCAGTGGCTACATGTGAGCTCAGACCTGGGTCTGCTGCTGTCT
GTCTTCCCAATATCCATGACCTTGACTGATGCAGGTGTCTAGGGATACGTCCATCCCCG
TCCTGCTGGAGCCCAGAGCACGGAAGCCTGGCCCTCCGAGGAGACAGAAGGGAGTGTCTG
GACACCATGACGAGAGCTTGGCAGAATAAATAAATCTTCTTTAAACAATTTTACGGCATGA
AGAAATCTGGACCAGTTTATTAAATGGGATTTCTGCCACAAACCTTGGAAGAATCACAT
CATCTTANNCCCAAGTGAAAATCTGTGTTGCGTAACAAAGAACATGACTGCGCTCCACAC
ATACATCATTTGCCCGGCGAGGCGGGACACAAGTCAACGACGGAACACTTGAGACAGGCC

```

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TACAACTGTGCACGGGTCAGAAGCAAGTTTAAGCCATACTTGCTGCAGTGAGACTACAT
TTCTGTCTATAGAAGATACCTGACTTGATCTGTTTTTCAGCTCCAGTTC CAGATGTGC
 ←----GTCAAGGGTCTACACG
GTGTTGTGGTCCCCAAGTATCACCTTCCAATTTCTGGGAG--→
CACAACACCAGGGGTTCATAGTGGAAGGTTAAAG-5'

CAGTGCTCTGGCCGGATCCTTGCCGCGCGGATAAAAACT---→

Confirmation of probe sequence

Following probe design, each probe sequence was confirmed by comparing the sequence against dbEST, the UniGene cluster set, and the assembled human genome using BLASTn at NCBI. Alignments, accession numbers, gi numbers, UniGene cluster numbers and names were examined and the most common sequence used for the probe. The final probe set was compiled into Table 8.

Example 22 - Production of an array of 8000 spotted 50mer oligonucleotides

We produced an array of 8000 spotted 50mer oligonucleotides. Examples 20 and 21 exemplify the design and selection of probes for this array.

Sigma-Genosys (The Woodlands, TX) synthesized un-modified 50-mer oligonucleotides using standard phosphoramidite chemistry, with a starting scale of synthesis of 0.05 μ mole (see, e.g., R. Meyers, ed. (1995) Molecular Biology and Biotechnology: A Comprehensive Desk Reference). Briefly, to begin synthesis, a 3' hydroxyl nucleoside with a dimethoxytrityl (DMT) group at the 5' end was attached to a solid support. The DMT group was removed with trichloroacetic acid (TCA) in order to free the 5'-hydroxyl for the coupling reaction. Next, tetrazole and a phosphoramidite derivative of the next nucleotide were added. The tetrazole protonates the nitrogen of the phosphoramidite, making it susceptible to nucleophilic attack. The DMT group at the 5'-end of the hydroxyl group blocks further addition of nucleotides in excess. Next, the inter-nucleotide linkage was converted to a phosphotriester bond in an oxidation step using an oxidizing agent and water as the oxygen donor. Excess nucleotides were filtered

out and the cycle for the next nucleotide was started by the removal of the DMT protecting group. Following the synthesis, the oligo was cleaved from the solid support. The oligonucleotides were desalted, resuspended in water at a concentration of 100 or 200 μM , and placed in 96-deep well format. The oligonucleotides were re-arrayed into Whatman Uniplate 384-well polypropylene V bottom plates. The oligonucleotides were diluted to a final concentration 30 μM in 1X Micro Spotting Solution Plus (Telechem/arrayit.com, Sunnyvale, CA) in a total volume of 15 μl . In total, 8,031 oligonucleotides were arrayed into twenty-one 384-well plates.

Arrays were produced on Telechem/arrayit.com Super amine glass substrates (Telechem/arrayit.com), which were manufactured in 0.1 mm filtered clean room with exact dimensions of 25x76x0.96 mm. The arrays were printed using the Virtek Chipwriter with a Telechem 48 pin Micro Spotting Printhead. The Printhead was loaded with 48 Stealth SMP3B TeleChem Micro Spotting Pins, which were used to print oligonucleotides onto the slide with the spot size being 110-115 microns in diameter.

Example 23- Amplification, labeling, and hybridization of total RNA to an oligonucleotide microarray

Amplification, labeling, hybridization and scanning

Samples consisting of at least 2 μg of intact total RNA were further processed for array hybridization. Amplification and labeling of total RNA samples was performed in three successive enzymatic reactions. First, a single-stranded DNA copy of the RNA was made (hereinafter, "ss-cDNA"). Second, the ss-cDNA was used as a template for the complementary DNA strand, producing double-stranded cDNA (hereinafter, "ds-cDNA, or cDNA"). Third, linear amplification was performed by in vitro transcription from a bacterial T₇ promoter. During this step, fluorescent-conjugated nucleotides were incorporated into the amplified RNA (hereinafter, "aRNA").

The first strand cDNA was produced using the Invitrogen kit (Superscript II). The first strand cDNA was produced in a reaction composed of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM MgCl₂ (1x First Strand Buffer, Invitrogen), 0.5 mM dGTP, 0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dCTP, 10 mM DTT, 10 U reverse transcriptase (Superscript II, Invitrogen, #18064014), 15 U RNase inhibitor (RNAGuard, Amersham

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Pharmacia, #27-0815-01), 5 μ M T7T24 primer

(5'-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTTTTT
TTTTTTTTTTTTT-3'), (SEQ ID NO.:8831) and 2 μ g of selected sample total RNA.

Several purified, recombinant control mRNAs from the plant *Arabidopsis thaliana* were added to the reaction mixture: 20 pg of CAB and RCA, 14 pg of LTP4 and NAC1, and 2 pg of RCP1 and XCP2 (Stratagene, #252201, #252202, #252204, #252208, #252207, #252206 respectively). The control RNAs allow the estimate of copy numbers for individual mRNAs in the clinical sample because corresponding sense oligonucleotide probes for each of these plant genes are present on the microarray. The final reaction volume of 40 μ l was incubated at 42°C for 60 min.

For synthesis of the second cDNA strand, DNA polymerase and RNase were added to the previous reaction, bringing the final volume to 150 μ l. The previous contents were diluted and new substrates were added to a final concentration of 20 mM Tris-HCl (pH 7.0) (Fisher Scientific, Pittsburgh, PA #BP1756-100), 90 mM KCl (Teknova, Half Moon Bay, CA, #0313-500), 4.6 mM MgCl₂ (Teknova, Half Moon Bay, CA, #0304-500), 10 mM (NH₄)₂SO₄ (Fisher Scientific #A702-500) (1x Second Strand buffer, Invitrogen), 0.266 mM dGTP, 0.266 mM dATP, 0.266 mM dTTP, 0.266 mM dCTP, 40 U *E. coli* DNA polymerase (Invitrogen, #18010-025), and 2 U RNaseH (Invitrogen, #18021-014). The second strand synthesis took place at 16°C for 120 minutes.

Following second-strand synthesis, the ds-cDNA was purified from the enzymes, dNTPs, and buffers before proceeding to amplification, using phenol-chloroform extraction followed by ethanol precipitation of the cDNA in the presence of glycogen. Alternatively, a silica-gel column is used to purify the cDNA (e.g. Qiaquick PCR cleanup from Qiagen, #28104). The cDNA was collected by centrifugation at >10,000 \times g for 30 minutes, the supernatant is aspirated, and 150 μ l of 70% ethanol, 30% water was added to wash the DNA pellet. Following centrifugation, the supernatant was removed, and residual ethanol was evaporated at room temperature.

Linear amplification of the cDNA was performed by in vitro transcription of the cDNA. The cDNA pellet from the step described above was resuspended in 7.4 μ l of water, and in vitro transcription reaction buffer was added to a final volume of 20 μ l

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containing 7.5 mM GTP, 7.5 mM ATP, 7.5 mM TTP, 2.25 mM CTP, 1.025 mM Cy3-conjugated CTP (Perkin Elmer; Boston, MA, #NEL-580), 1x reaction buffer (Ambion, Megascript Kit, Austin, TX and #1334) and 1 % T₇ polymerase enzyme mix (Ambion, Megascript Kit, Austin, TX and #1334). This reaction was incubated at 37°C overnight. Following in vitro transcription, the RNA was purified from the enzyme, buffers, and excess NTPs using the RNeasy kit from Qiagen (Valencia, CA; # 74106) as described in the vendor's protocol. A second elution step was performed and the two eluates were combined for a final volume of 60 µl. RNA is quantified using an Agilent 2100 bioanalyzer with the RNA 6000 nano LabChip.

Reference RNA was prepared as described above, except that 10 µg of total RNA was the starting material for amplification, and Cy5-CTP was incorporated instead of Cy3CTP. Reference RNA from five reactions was pooled together and quantitated as described above.

Hybridization to an array

RNA was prepared for hybridization as follows: for an 18mm×55mm array, 20 µg of amplified RNA (aRNA) was combined with 20 µg of reference aRNA. The combined sample and reference aRNA was concentrated by evaporating the water to 5 µl in a vacuum evaporator. Five µl of 20 mM zinc acetate was added to the aRNA and the mix incubated at 60°C for 10 minutes to fragment the RNA into 50-200 bp pieces. Following the incubation, 40 µl of hybridization buffer was added to achieve final concentrations of 5×SSC and 0.20 %SDS with 0.1 µg/ul of Cot-1 DNA (Invitrogen) as a competitor DNA. The final hybridization mix was heated to 98°C, and then reduced to 50°C at 0.1°C per second.

Alternatively, formamide is included in the hybridization mixture to lower the hybridization temperature.

The hybridization mixture was applied to the microarray surface, covered with a glass coverslip (Corning, #2935-246), and incubated in a humidified chamber (Telechem, AHC-10) at 62°C overnight. Following incubation, the slides were washed in 2×SSC, 0.1% SDS for two minutes, then in 2×SSC for two minutes, then in 0.2×SSC for two

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minutes. The arrays were spun at 1000×g for 2 minutes to dry them. The dry microarrays are then scanned by methods described above.

Example 24: Analysis of Human Transplant Patient Mononuclear cell RNA Hybridized to a 24,000 Feature Microarray.

Patients who had recently undergone cardiac transplant and were being monitored for rejection by biopsy were selected and enrolled in a clinical study, as described in Example 11. Blood was drawn from several patients and mononuclear cells isolated as described in Example 8. The rejection grade determined from the biopsy is presented in Table 9 for some of the patient samples. Four samples (14-0001-2, 14-0001-3, 14-0005-1 and 14-0005-2) from one center were selected for further examination. Two sets of paired samples were available that allowed comparison of severe rejection (rejection grade 3A) to minimal or no rejection (rejection grade 1 or 0). These two groups are designated "high rejection grade" and "low rejection grade", respectively.

Additional RNA was isolated from the mononuclear cells of enrolled cardiac allograft recipients as described in Example 8. The yield of RNA from 8 ml of blood is shown in Table 9, below.

1 or 2 µg of total RNA was amplified by making cDNA copies using a T7T24 primer and subsequent in vitro transcription, as described in Example 23. This "target" amplified RNA was labeled by incorporation of Cy3-conjugated nucleotides, as described in Example 23. The amplified RNA was quantified by analysis at A260 on a spectrophotometer.

Hybridization to the 8,000 probe (24,000-feature) microarray (described in Examples 20-22) was performed essentially as described in Example 23. 20 µg of amplified and labeled RNA was combined with 20 µg of R50 reference RNA that was labeled and prepared as described in Example 9.

The sample and reference amplified and labeled RNAs were combined and fragmented at 95°C for 30 min, as described in Example 23. The fragmented RNA was mixed with 40 µl of hybridization solution (to bring the total to 50 µl) and applied to the 8,000-probe, 24,000-feature microarray and covered with a 21mm×60mm coverslip. The arrays were hybridized overnight and washed as described in Example 23.

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Once hybridized and washed, the arrays were scanned as described in Example 23. The full image produced by the Agilent scanner G2565AA was flipped, rotated, and split into two images (one for each signal channel) using TIFFSplitter (Agilent, Palo Alto, CA). The two channels are the output at 532 nm (Cy3-labeled sample) and 633 nm (Cy5-labeled R50). The individual images were loaded into GenePix 3.0 (Axon Instruments, Union City, CA) and the software was used to determine the median pixel intensity for each feature (F_i) and the median pixel intensity of the local background for each feature (B_i) in both channels. The standard deviation (SDF_i and SDB_i) for each is also determined. Features for which GenePix could not discriminate the feature from the background were "flagged", and the data were deleted from further consideration.

From the remaining data, the following calculations were performed.

The first calculation performed was the signal to noise ratio:

$$S/N = \frac{F_i - B_i}{SDB_i}$$

All features with a S/N less than 3 in either channel were removed from further consideration. All features that did not have GenePix flags and passed the S/N test were considered usable features. The background-subtracted signal (hereinafter, "BGSS") was calculated for each usable feature in each channel ($BGSS_i = F_i - B_i$).

The BGSS was used for the scaling step within each channel. The median BGSS for all usable features was calculated. The $BGSS_i$ for each feature was divided by the median BGSS. The median BGSS for the scaled data then became 1 for each channel on each array. This operation did not change the distribution of the data, but did allow each to be directly compared

The scaled $BGSS_i$ (S_i) for each feature was used to calculate the ratio of the Cy3 to the Cy5 signal:

$$R_n = \frac{Cy3S_i}{Cy5S_i}$$

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The ratio data from the triplicate features were combined for each probe on the array. If all three features were still usable, their average was taken (R_p) and the coefficient of variation (hereinafter "CV") was determined. If the CV was less than 15%, the average was carried forward for that probe. If the CV was greater than 15% for the triplicate features, then the average of the two features with the closest R_n values were used. If there were only two usable features for a given probe, the average of the two features was used. If there was only one usable feature for a given probe, the value of that feature was used.

The logarithm of the average ratio was taken for each probe ($\log R_p$). This value was used for comparison among arrays. For comparison of gene expression in high rejection grade patients to gene expression from low rejection grade patients, the average was taken for each probe for hybridizations 107739 and 107741 (high rejection grades) and 107740 and 107742 (low rejection grades). Since there were only two patients, each with a change from high to low rejection grade, there should be less variability in the data than if all four samples were from different patients. The results of this comparison were plotted in Figure 9. The X-axis is the high rejection grade average (the average of each probe for hybridizations of samples from high rejection grade patients) and the Y-axis is the low rejection grade average. There was complete data for 5562 probes, all plotted in Figure 9. Each "point" in the graph corresponded to a probe on the microarray.

A "cluster" of points were shaded in white. Points within the cluster represented genes with expression that is not significantly changed from one sample group to the other. The far ends of the cluster corresponded to genes that are expressed at either low or high levels in each group.

Outlier points, corresponding to genes with differential expression between high and low rejection grade patients, were shaded black and are further described in Table 10. There was one point above the cluster (indicating that expression was relatively higher in the low rejection grade than in the low rejection grade), and 7 points below the cluster (indicating that expression was relatively higher in the high rejection grade than in the low rejection grade).

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Many of the differentially expressed genes had unknown or poorly described functions. One, corresponding to probe number 8091, was known in the public databases only as a predicted mRNA and protein.

Using the data from samples 107739 (Grade 3A rejection) and 107742 (Grade 0), a scaled ratio of sample (Cy3) to reference (Cy5) expression was determined using the same techniques. The ratio of was taken of these scaled ratios, denoted "the ratio of scaled ratios (hereinafter, "SR"). Replicate features were not combined and all probes with $S/N < 3$ in either channel were filtered out. Some probes with differential expression between these two samples are shown in Figure 10. In this Figure, the probes are sorted from the top to the bottom by relative expression in the first grade 0 sample vs grade 3A (ratio of SRs, grade 0/3A).

Diagnostic accuracy for sample classification is determined using additional samples and suitable methods for correlation analysis.

Comparing Figure 10 and Table 10, genes of particular interest include those corresponding to SEQ ID NO:2476, SEQ ID NO: 2407, SEQ ID NO:2192, SEQ ID NO: 2283, SEQ ID NO:6025, SEQ ID NO: 4481, SEQ ID NO:3761, SEQ ID NO: 3791, SEQ ID NO:4476, SEQ ID NO: 4398, SEQ ID NO:7401, SEQ ID NO: 1796, SEQ ID NO:4423, SEQ ID NO: 4429, SEQ ID NO:4430, SEQ ID NO: 4767, SEQ ID NO:4829 and SEQ ID NO: 8091.

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Table 1

Disease Classification	Disease/Patient Group
Cardiovascular Disease	Atherosclerosis Unstable angina Myocardial Infarction Restenosis after angioplasty Congestive Heart Failure Myocarditis Endocarditis Endothelial Dysfunction Cardiomyopathy Cardiovascular drug use
Endocrine Disease	Diabetes Mellitus I and II Thyroiditis Addisson's Disease
Infectious Disease	Hepatitis A, B, C, D, E, G Malaria Tuberculosis HIV Pneumocystis Carinii Giardia Toxoplasmosis Lyme Disease Rocky Mountain Spotted Fever Cytomegalovirus Epstein Barr Virus Herpes Simplex Virus Clostridium Difcile Colitis Meningitis (all organisms) Pneumonia (all organisms) Urinary Tract Infection (all organisms) Infectious Diarrhea (all organisms) Anti-infectious drug use
Angiogenesis	Pathologic angiogenesis Physiologic angiogenesis Treatment induced angiogenesis Pro or anti-angiogenic drug use
Inflammatory/Rheumatic	Rheumatoid Arthritis Systemic Lupus Erythematosus Sjogrens Disease CREST syndrome Scleroderma Ankylosing Spondylitis Crohn's Ulcerative Colitis Primary Sclerosing Cholangitis

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Table 1 (continued)

Disease Classification	Disease/Patient Group
Inflammatory/Rheumatic	Appendicitis Diverticulitis Primary Biliary Sclerosis Wegener's Granulomatosis Polyarteritis nodosa Whipple's Disease Psoriasis Microscopic Polyangitis Takayasu's Disease Kawasaki's Disease Autoimmune hepatitis Asthma Churg-Strauss Disease Beurger's Disease Raynaud's Disease Cholecystitis Sarcoidosis Asbestosis Pneumoconioses Antinflammatory drug use
Transplant Rejection	Heart Lung Liver Pancreas Bowel Bone Marrow Stem Cell Graft versus host disease Transplant vasculopathy Skin Cornea Immunosuppressive drug use
Malignant Disorders	Leukemia Lymphoma Carcinoma Sarcoma
Neurological Disease	Alzheimer's Dementia Pick's Disease Multiple Sclerosis Guillain Barre Syndrome Peripheral Neuropathy

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We claim:

1. A system for detecting gene expression comprising at least two isolated DNA molecules wherein each isolated DNA molecule detects expression of a gene wherein said gene is selected from the group of genes corresponding to the oligonucleotides depicted in SEQ ID NO:1 - SEQ ID NO: 8143.
2. The system of claim 1 wherein said gene is selected from the group of genes corresponding to the oligonucleotides depicted in SEQ ID NO:2476, SEQ ID NO: 2407, SEQ ID NO:2192, SEQ ID NO: 2283, SEQ ID NO:6025, SEQ ID NO: 4481, SEQ ID NO:3761, SEQ ID NO: 3791, SEQ ID NO:4476, SEQ ID NO: 4398, SEQ ID NO:7401, SEQ ID NO: 1796, SEQ ID NO:4423, SEQ ID NO: 4429, SEQ ID NO:4430, SEQ ID NO: 4767, SEQ ID NO:4829, and SEQ ID NO: 8091.
3. The system of claim 1 wherein the DNA molecules are synthetic DNA, genomic DNA, PNA or cDNA.
4. The system of claim 1 wherein the isolated DNA molecules are immobilized on an array.
5. The system of claim 4 wherein the array is selected from the group consisting of a chip array, a plate array, a bead array, a pin array, a membrane array, a solid surface array, a liquid array, an oligonucleotide array, polynucleotide array or a cDNA array, a microtiter plate, a membrane and a chip.
6. A method of detecting gene expression comprising a) isolating RNA and b) hybridizing said RNA to the isolated DNA molecules of claim 1.
7. A method of detecting gene expression comprising a) isolating RNA; b) converting said RNA to nucleic acid derived from the RNA and c) hybridizing said nucleic acid derived from the RNA to the isolated DNA molecules of claim 1.
8. The method of claim 7 wherein said nucleic acid derived from the RNA is cDNA.

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9. A method of detecting gene expression comprising a) isolating RNA; b) converting said RNA to cRNA or aRNA and c) hybridizing said cRNA or aRNA to the isolated DNA molecules of claim 1.
10. A candidate library comprising at least two isolated oligonucleotides wherein the oligonucleotides have nucleotide sequences having at least 40-50, 50-60, 70-80, 80-85, 85-90, 90-95 or 95-100% sequence identity to the nucleotide sequences selected from the group consisting of SEQ ID NO:1- SEQ ID NO: 8143.
11. The candidate library of claim 10, wherein the nucleotide sequence comprises deoxyribonucleic acid (DNA) sequence, ribonucleic acid (RNA) sequence, synthetic oligonucleotide sequence, protein nucleic acid (PNA) sequence or genomic DNA sequence.
12. The candidate library of claim 11, wherein the candidate library is immobilized on an array.
13. The candidate library of claim 12, wherein the array is selected from the group consisting of: a chip array, a plate array, a bead array, a pin array, a membrane array, a solid surface array, a liquid array, an oligonucleotide array, polynucleotide array or a cDNA array, a microtiter plate, a membrane and a chip.
14. A diagnostic oligonucleotide for a disease comprising an oligonucleotide wherein the oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID NO:1 - SEQ ID NO: 8143 wherein said oligonucleotide detects expression of a gene that is differentially expressed in leukocytes in an individual with at least one disease criterion for at least one leukocyte-related disease compared to the expression of said gene in an individual without the at least one disease criterion, wherein expression of the gene is correlated with the at least one disease criterion.
15. The diagnostic oligonucleotide of claim 14, wherein the nucleotide sequence comprises DNA, cDNA, PNA, genomic DNA, or synthetic oligonucleotides.

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16. The diagnostic oligonucleotide of claim 14, wherein the disease criterion comprises data wherein the data is selected from physical examination data, laboratory data, patient historic, diagnostic, prognostic, risk prediction, therapeutic progress, and therapeutic outcome data.
17. The diagnostic oligonucleotide of claim 14, wherein the leukocytes comprise peripheral blood leukocytes or leukocytes derived from a non-blood fluid.
18. The diagnostic oligonucleotide of claim 17, wherein the non-blood fluid is isolated from the colon, sinus, esophagus, small bowel, pancreatic duct, biliary tree, ureter, vagina, cervix uterus, nose, ear, urethra, eye, open wound, abscess, stomach, cerebral spinal fluid, peritoneal fluid, pleural fluid, synovial fluid, bone marrow and pulmonary lavage.
19. The diagnostic oligonucleotide of claim 14, wherein the leukocytes comprise leukocytes derived from urine or a biopsy sample.
20. The diagnostic oligonucleotide of claim 14, wherein the leukocytes are peripheral blood mononuclear cells or T-lymphocytes.
21. The diagnostic oligonucleotide of claim 14, wherein the disease is selected from the group consisting of cardiac allograft rejection, kidney allograft rejection, liver allograft rejection, atherosclerosis, congestive heart failure, systemic lupus erythematosus (SLE), rheumatoid arthritis, osteoarthritis, and cytomegalovirus infection.
22. The diagnostic oligonucleotide of claim 14, wherein the differential expression is one or more of: a relative increase in expression, a relative decrease in expression, presence of expression or absence of expression.
23. A diagnostic agent comprising an oligonucleotide wherein the oligonucleotide has a nucleotide sequence selected from the group consisting of SEQ ID NO:1 - SEQ ID NO: 8143 wherein said oligonucleotide detects expression of a gene that is differentially expressed in leukocytes in an individual over time.

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24. The agent of claim 23 wherein said oligonucleotide is selected from the group consisting of SEQ ID NO:2476, SEQ ID NO: 2407, SEQ ID NO:2192, SEQ ID NO:2283, SEQ ID NO:6025, SEQ ID NO:4481, SEQ ID NO:3761, SEQ ID NO:3791, SEQ ID NO:4476, SEQ ID NO:4398, SEQ ID NO:7401, SEQ ID NO:1796, SEQ ID NO:4423, SEQ ID NO:4429, SEQ ID NO:4430, SEQ ID NO:4767, SEQ ID NO:4829, and SEQ ID NO:8091.

25. A diagnostic probe set for a disease comprising at least two probes wherein each probe detects expression of a gene wherein the gene is selected from the group of genes corresponding to the oligonucleotides depicted in SEQ ID NO: 1 - SEQ ID NO:8143 wherein each gene is differentially expressed in leukocytes in an individual with at least one disease criterion for a disease selected from Table 1 as compared to the expression of the gene in leukocytes in an individual without the at least one disease criterion, wherein expression of the gene is correlated with the at least one disease criterion.

26. An isolated nucleic acid wherein said nucleic acid comprises a sequence depicted in SEQ ID NO:8144 - SEQ ID NO:8766.

27. An expression vector containing the nucleic acid of claim 26 in operative association with a regulatory element which controls expression of the nucleic acid in a host cell.

28. A host cell comprising the expression vector of claim 27.

29. The host cell of claim 27, wherein the host cell is a prokaryotic cell or a eukaryotic cell.

30. A kit comprising the system of claim 1.

31. A system for detecting gene expression in leukocytes comprising an isolated DNA molecule wherein said isolated DNA molecule detects expression of a gene wherein said gene is selected from the group of genes corresponding to the oligonucleotides depicted in SEQ ID NO: 1-SEQ ID NO: 8143 and said gene is differentially expressed in said leukocytes in an individual with at least one disease

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criterion for a disease selected from Table 1 compared to the expression of said gene in leukocytes in an individual without the at least one disease criterion.

32. The system of claim 31 wherein the DNA molecule is at least 16 nucleotides in length.

33. The system of claim 31 wherein the DNA molecules are synthetic DNA, genomic DNA, PNA or cDNA.

34. The system of claim 31 wherein the isolated DNA molecule is immobilized on an array.

35. The system of claim 34 wherein the array is selected from the group consisting of a chip array, a plate array, a bead array, a pin array, a membrane array, a solid surface array, a liquid array, an oligonucleotide array, polynucleotide array or a cDNA array, a microtiter plate, a membrane and a chip.

36. A method of detecting gene expression comprising a) isolating RNA and b) hybridizing said RNA to the isolated DNA molecule of claim 31.

37. A method of detecting gene expression comprising a) isolating RNA; b) converting said RNA to nucleic acid derived from the RNA and c) hybridizing said nucleic acid derived from said RNA to the isolated DNA molecules of claim 31.

38. The method of claim 37 wherein said nucleic acid derived from the RNA is cDNA.

39. A method of detecting gene expression comprising a) isolating RNA; b) converting said RNA to cRNA or aRNA and c) hybridizing said cRNA or aRNA to the isolated DNA molecule of claim 31.

40. A method of diagnosing a disease comprising obtaining a leukocyte sample from an individual, contacting said leukocyte sample with the gene expression system of claim 31 and comparing the expression of the gene with a molecular signature indicative of the presence or absence of said disease.

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41. A method of monitoring progression of a disease comprising: obtaining a leukocyte sample from an individual, contacting said leukocyte sample with the gene expression system of claim 31, and comparing the expression of the gene with a molecular signature indicative of the presence or absence of disease progression.

42. A method of monitoring the rate of progression of a disease comprising: obtaining a leukocyte sample from an individual, contacting said leukocyte sample with the gene expression system of claim 31, and comparing the expression of the gene with a molecular signature indicative of the presence or absence of disease progression.

43. A method of predicting therapeutic outcome comprising: obtaining a leukocyte sample from an individual, contacting said leukocyte sample with the gene expression system of claim 31, and comparing the expression of the gene with a molecular signature indicative of the predicted therapeutic outcome.

44. A method of determining prognosis for a patient comprising obtaining a leukocyte sample from a patient, contacting said leukocyte sample with the gene expression system of claim 31, and comparing the expression of the gene, and comparing the expression of the gene with a molecular signature indicative of the prognosis.

45. A method of predicting disease complications in an individual comprising obtaining a leukocyte sample from an individual, contacting said leukocyte sample with the gene expression system of claim 31, and comparing the expression of the gene with a molecular signature indicative of the presence or absence of disease complications.

46. A method of monitoring response to treatment in an individual, comprising obtaining a leukocyte sample from an individual, contacting said leukocyte sample with the gene expression system of claim 31, and comparing the expression of the gene with a molecular signature indicative of the presence or absence of response to treatment.

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47. The method according to claim 46, wherein said method further comprises characterizing the genotype of the individual, and comparing the genotype of the individual with a diagnostic genotype, wherein the diagnostic genotype is correlated with at least one disease criterion.

48. The method according to claim 41, wherein said method further comprises characterizing the genotype of the individual, and comparing the genotype of the individual with a diagnostic genotype, wherein the diagnostic genotype is correlated with at least one disease criterion.

49. The method according to claim 42, wherein said method further comprises characterizing the genotype of the individual, and comparing the genotype of the individual with a diagnostic genotype, wherein the diagnostic genotype is correlated with at least one disease criterion.

50. The method according to claim 43, wherein said method further comprises characterizing the genotype of the individual, and comparing the genotype of the individual with a diagnostic genotype, wherein the diagnostic genotype is correlated with at least one disease criterion.

51. The method according to claim 44, wherein said method further comprises characterizing the genotype of the individual, and comparing the genotype of the individual with a diagnostic genotype, wherein the diagnostic genotype is correlated with at least one disease criterion.

52. The method of claim 50, wherein the genotype is analyzed by one or more methods selected from the group consisting of Southern analysis, RFLP analysis, PCR, single stranded conformation polymorphism, and SNP analysis.

53. A method of RNA preparation suitable for diagnostic expression profiling comprising: obtaining a leukocyte sample from a subject, adding actinomycin-D to a final concentration of 1 ug/ml, adding cycloheximide to a final concentration of 10 ug/ml, and extracting RNA from the leukocyte sample.

54. The method of claim 52, wherein the actinomycin-D and cycloheximide are present in a sample tube to which the leukocyte sample is added.